CHARACTERIZING THE FATE AND TRANSPORT OF CHEMICALS OF EMERGING CONCERN (CECs) FROM SWINE MANURE DURING WASTE TO ENERGY PROCESSES

BY

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DISSERTATION

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ABSTRACT

Animal manure and domestic wastewater contain chemicals of emerging concern (CECs) that can threaten ecosystems and human health. For instance, estrone (E1) and 17β-estradiol (E2) can disrupt the reproductive biology of vertebrates at very low concentrations. This study investigates the effects of novel manure management systems that can simultaneously produce bioenergy and reduce the discharge of CECs to improve energy security and water quality.

Natural occurrence of estrogenic hormones of swine manure from three different swine barns were investigated using a serial extraction method. Daily production of total hormones per pig were as follows: farrowing (1,333 ± 19 μg/day-hd) > gestation (789 ± 15 μg/day-hd) > finishing (518 ± 5 μg/day-hd). Sedimentation, screening, and filtration processes were used to remove the solids content (up to 98%) and produce a liquid portion of animal manure (LPAM) which was used as an influent for biological processes that cultivated bacteria and algae to provide improved LPAM water quality and a bioenergy feedstock. The mixed algal-bacterial bioreactor (MABB) was operated with and without the addition of granular activated carbon (GAC) to enhance the removal of CECs and other organics from the LPAM. The resulting biomass was harvested for biofuel conversions via hydrothermal liquefaction (HTL) and catalytic hydrothermal gasification (CHG), which were performed under 16 different conditions to study the effects of hydrothermal processes on the removal of bioactive CECs. Solid Phase Extraction (SPE) of estrogenic hormones and hydrolysis of florfenicol (FF) followed by GC/MS analysis was used to measure the concentration of CECs before and after algal bioreactor treatment and hydrothermal conversion processes.

The research demonstrated that the algal manure treatment and thermochemical-waste-to-energy processes could simultaneously remove the CECs from animal manure by 96.5% to 99.9%

in total estrogenic hormones and 93.0% to 99.9% in antibiotics, while converting biomass into biocrude oil with up to a 40.0% yield (dry basis) and a bio-char solid residue with a 12.0% yield (dry basis) using the HTL. In addition, the CHG process converted the same biomass to syn-gas with an average yield of up to 54.4% (dry basis). The most favorable process conditions for both bioenergy production and hormones removal were 300°C/60minutes for HTL and 500°C/60minutes with Ruthenium catalyst (Ru) for CHG. The Xenoscreen YES assay was used to investigate the effects hydrothermal processes on eliminating the estrogenic activity of LPAM. Increasing reaction temperature in hydrothermal processes generally reduced the estrogenic potency and the concentration of residual hormone compounds. Antibiotic resistant bacteria (ARB) assay tests were conducted with HTL-WW and CHG-WW to determine if HTL and CHG can reduce the capacity of bacterial cells to generate antibiotic resistance. Despite the complex matrix of wastewaters from hydrothermal processes, the ARB assay was sensitive in measuring the antibiotic resistance, and the HTL and CHG processing could eliminate the capacity of samples to generate bacteria with resistance to the target antibiotic, FF.

Water quality parameters such as soluble COD, TN, TP, NH_3-N in the effluents from various bioreactor treatments were investigated to demonstrate the ability to extract bioactive CECs and other dissolved organics from LPAM, while also creating a feedstock for bioenergy production. The percent removal of soluble COD, TN, TP, NH_3-N in MABB with GAC were 74.6, 30.1, 39.5, and 97.0%, respectively. Mammalian cell cytotoxicity assays using Chinese hamster ovary (CHO) cells were conducted using LPAM, bioreactor effluents from a MABB and a conventional activated sludge reactor (CAS), as well as the effluent wastewaters after HTL (HTL-WW) and CHG conversions (CHG-WW). In this study, adding GAC was synergistic with MABB and CAS in reducing the cytotoxicity of LPAM by adsorption of toxic compounds and/or stabilized performance of each bioreactor. More importantly, HTL-WW and CHG-WW exhibited the lowest

cytotoxic characteristics under the optimal conditions for bioenergy production, 300°C/60minutes for HTL and 500°C/60minutes with Ruthenium catalyst (Ru) for CHG. To quantify the acute toxicity of wastewater samples, a Microtox® assay was also conducted. MABB with GAC showed the lowest acute toxicity due to slightly better organics removal via adsorption and higher temperature. The acute toxicities of the HTL-WW and CHG-WW demonstrated a similar trend as the corresponding CHO cell cytotoxic data, and exhibited the lowest acute toxicity under the optimal conditions for HTL and CHG processes.

In the MABB with GAC, the average percent distribution of heavy metals to biomass and effluents were 6.1 and 80.4%, respectively. After HTL and CHG treatment of biomass, the highest concentration of heavy metals was found mostly in the solids residue (84.2 and 73.1%, respectively), followed by the aqueous product (10.4 and 20.8%, respectively), with only a miniscule fraction in the biocrude oil (0.1 and 2.2%, respectively). The concentrations of arsenic (As), lead (Pb), copper (Cu), zinc (Zn), and cadmium (Cd) ranged from 0.02 to 37.3 mg/L in the aqueous phase after HTL and CHG tests, which are higher than the typical limits allowed for livestock drinking water and irrigation. (Ayers et al., 1985; U.S.EPA. 1974). However, if these hydrothermal process aqueous products were blended back with the separated water fraction of raw manure, the combined concentration would be below recommended heavy metal limits.

In conclusion, these findings will contribute to the development of cost-effective systems to increase bioenergy production, reduce water pollution, and enhance opportunities for the treatment and reuse of the aqueous fraction of livestock manure.

To Mother

For your love, support and understanding

and

To Wife Jieun Lee and Sons, Yunegeon Shin and Andrew Shin

Who love me, and whom I dearly love.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance pairwise test
APHA	American Public Health Association
ARB	Antibiotic resistance bacteria
ARG	Antibiotic resistance gene
BOD	Biological Oxygen Demand
BPA	Bisphenol A
CAFO	Concentrated Animal Feeding Operations
CAS	Conventional Activated Sludge reactor
CECs	Chemicals of Emerging Concern
CF	Ceftiofur
CHG	Catalytic Hydrothermal Gasification
CHO	Chinese Hamster Ovary
COD	Chemical Oxygen Demand
CPRG	Chromogenic substrate chlorophenol red-β-D-galactopyranoside
DMSO	Demethylsulfoxide
DON	Dissolved Organic Nitrogen
E1	Estrone
E2	17β – Estradiol
E3	Estriol
EE2	17α – Estradiol
EEQ	Estradiol equivalent

FDA	Food and Drug Administration
FF	Florfenicol
4-MASP	4’-(methylsulfonyl)acetophenone
4-MSB	4-(methylsulfonlyl)benzaldehyde
GAC	Granular Activated Carbon
GC/MS	Gas Chromatography /Mass Spectrometry
HPLC/MS	High Performance Liquid Chromatography/ Mass Spectrometry
HRT	Hydraulic retention time
HTL	Hydrothermal Liquefaction
LPAM	Liquid portion of animal manure
MABB	Mixed Algal – Bacterial Bioreactor
MPS	methyl phenyl sulfone
NOCs	Nitrogenous Organic Compounds
OD680	Optical Density at 680 nm
PAC	Powered activated carbon
SPE	Solid Phase Extraction
SRT	Solid retention time
TN	Total Nitrogen
TSS	Total Suspended Solid
UCSD	Urbana-Champaign Sanitary District
WHO	World Health Organization
YES	Yeast estrogen screen

CHAPTER 1. INTRODUCTION

1.1 BACKGROUND

The occurrence of bioactive contaminants such as antibiotics and hormones associated with concentrated animal feeding operations (CAFOs) is an important issue highlighted in many previous studies (Andaluri et al., 2012; Brooks & McLaughlin. 2009; Chee-Sanford et al., 2001; Chee-Sanford et al., 2009; Raman et al., 2001; Raman et al., 2004). According to a report from the U.S. Food and Drug Administration (FDA), the use of antibiotics in livestock animals has been increasing, and roughly 70% of the total volume of antibiotics produced in the USA were used in the food-animal industry to prevent, control, treat disease, and to promote the growth of livestock animals (FDA. 2004).

Since the 1950s, several steroid hormone drugs, including natural estrogen, progesterone, and their synthetic versions, have been approved by the Food and Drug Administration (FDA) for promoting the growth rate of livestock. The increased concentrations of estrogenic hormones are excreted with natural steroidal estrogen hormones in the urine and feces of the livestock, and the total excretion of 17β-estradiol and estrone are several milligrams per day per animal (Hanselman et al., 2003; Lange et al., 2002; Nichols et al., 1997; Peterson et al., 2008; Ternes et al., 1999a; Ternes et al., 1999b). According to USDA, (2011), animal feeding operations generate more than 135 million tons of dry matter per year, and the annual level of estrogen excretion by livestock animals is approximately 39 tons in the European Union and 41 tons in the United States (Lange et al., 2002). Excreted estrogenic compounds can enter the environment in a variety of ways including runoff into surface waters after field applications as well as leaching from holding tanks and composting facilities (Hanselman et al., 2003; Lange et al., 2002). If surface and groundwater

resources are contaminated by these estrogenic compounds, the reproduction and development of wildlife species in the aquatic environment can be adversely affected as a result of endocrine disruption which can occur at 1 ng/L concentrations of 17β-estradiol and estrone (Irwin et al., 2001; Jobling et al., 1998; Purdom et al., 1994; Thorpe et al., 2003). Estrogen concentrations in agricultural runoffs are three to four times higher than municipal wastewater (Shore et al., 1993). Therefore, it is particularly important to recognize the fate and transport of these bioactive compounds in animal farming systems, and utilize integrated manure management systems that cost-effectively mitigate the associated risks.

The routine use of antibiotics to promote growth can be particularly problematic because it can lead to antibiotic resistant bacteria over time. A 2013 report from the Center for Disease Control and Prevention (CDC) states that at least 2 million people become infected with antibiotic-resistant bacteria every year and at least 23,000 people die as a direct result of these infections (Ventola. 2015). There are several major factors exacerbating antibiotic resistance, and the use of antibiotics in the livestock industry is one such major factor contributing to illness (Ventola. 2015).

Antibiotics and hormones are common bioactive CECs which are frequently released into the environment as a by-product of human and livestock waste. Significant public concerns are raised because of the potential of pathogenic microorganisms increasing in antibiotic resistance which may disrupt the endocrine processes of both humans and animals. Therefore, a way to clearly understand the fate and transport of CECs from manure management systems is critical to minimize deleterious effects. A system which can simultaneously reduce the hazards associated with bioactive compounds present in animal waste and convert waste into an energy resource is being investigated. In addition, some process enhancements which target improved extraction rates of CECs from liquid portions of manure will be evaluated, primarily including runoff transport and irrigation pathways. We specifically propose an investigation and evaluation of the feasibility

of biological treatments with adsorbents to capture dissolved organics and bioactive CECs which can subsequently be subjected to HTL or CHG for bioenergy production. These methods can convert a concentrated organic mixture including CECs into various forms of bioenergy while simultaneously deactivating a wide spectrum of bioactive compounds, providing a solution to prevent the spread of antibiotic resistance and producing an alternative source of bioenergy. The bioenergy production using the concentrated organic waste is particularly important as it contributes towards another environmental issue associated with lowering greenhouse gas emissions and provides an economic reason to offset the cost of subjecting waste to these treatment methods.

1.2 STUDY FOR THE IMPROVED SUSTAINABILITY IN US AGRICULTURE

In this study, an integrated manure management system was proposed, which can provide an effective method to stop the transport of CECs by capturing them near its source and converting its components into value-added co-products before being released into the environment. The integrated manure management system improves the sustainability of United States agriculture systems through the elimination of potential negative impacts of releasing CECs from CAFOs into the environment which include human and animal pathogens becoming more inclined towards antibiotic resistance as well as the feminization of aquatic life. In addition, this study addresses an intent to improve the efficiency of water reuse using wastewater sources to produce bioenergy while simultaneously improving the quality of those water resources for utilization elsewhere. This proposed alternative approach in the management of manure can significantly increase environmental sustainability through the reduction of the release of undesirable residuals from livestock manure. The production of novel bioenergy resources ultimately fosters energy security

and reduces the total volume of greenhouse gas emissions. This research is economically friendly as it provides a new useful bioenergy product which can be derived from livestock wastewater.

If applied towards large CAFOs, the integrated manure management system can beneficially impact waste management. In the United States, the readily collectable volume of human, animal, and food waste exceeds 200 million tons of dry solids every year and the largest source of biosolid waste is manure (USDA, 2011; USEPA, 2008). These solids contain significant volumes of organic carbon and nitrogen that, in current manure treatment systems, are transformed by microorganisms and released into the atmosphere as greenhouse gases such as CH_4, N_2O and CO_2. The integrated system in this study would instead transform carbon into biogas and biocrude oil products which could ideally replace fossil fuels, treat antibiotics, estrogenic compounds, and other important bioactive CECs from livestock wastewater. Through this transformation, CECs are converted from a potentially liable compound into a favorable bioenergy product. This proposal seeks to reduce the emission of CECs into the environment, subsequently making a significant contribution towards the improvement of health (fewer antibiotic-resistant infections) and amelioration of ecosystem services (fewer instances of the feminization of male aquatic organisms).

A better understanding of the fundamental processes describing the fate of CECs in manure from waste-to-energy systems can help in sustaining ecosystem services by evading the destabilizing impacts of releasing CECs associated with typical agricultural activities. Specifically, the fundamental process research will determine how to minimize or even entirely eradicate the release of endocrine disrupting hormones from livestock feed operations because discharges of CECs into aquatic environment can upset the natural growth and reproduction of aquatic ecosystems which provide water quality enhancements in well-balanced ecosystems. Reduced discharges of antibiotics would instead minimize the proliferation of antibiotic resistance which

would subsequently curtail the assault of resistant pathogens stemming from the ecosystem. Additionally, reducing the selective pressures emanating from the presence of antibiotics contributes towards increased diversity and robustness of environmental microbial assemblages which yields the breakdown of waste products derivative of managed agroecosystems.

1.3 SCIENTIFIC CONTRIBUTIONS AND NEW IDEAS

This integrated management system provides a new alternative to conventional manure management systems that is influenced by nature and applied biology, ecology, and engineering principles to sustain waste-to-energy systems, harmonizing both energy and environmental goals. This alliance synergistically integrates our infrastructure for both bioenergy production and waste treatment to simultaneously produce biofuel resources, lessen carbon emissions, and clean wastewater. Energy sustainability and the improvement of the quality of available water resources can be achieved through this integrated system.

The advancement of the fundamental engineering of waste-to-energy systems as well as the effects of bioactive CECs are being investigated to maintain healthy, sustainable agroecosystems. The efficacy of different combinations of wastewater treatment processes as well as thermochemical conversation processes on the elimination and transformation of crucial CECs will be discussed in new data provided by various experiments. A comprehensive process design and procedure of manure management systems which harmonize the needs for wastewater treatment and energy production will be supported by operating the mixed algal bioreactor and hydrothermal waste-to-energy processes.

1.4 EFFECTS OF BIOACTIVE CECS ON HUMAN HEALTH AND ENVIRONMENT

Numerous human and livestock sources have produced wastewater discharge which contain various bioactive CECs such as steroidal estrogens, pharmaceuticals, plasticizers, surfactants and other chemicals (Brooks & McLaughlin. 2009; Chee-Sanford et al., 2009; Osada et al., 2006; Pals & Plewa. 2015). Various studies recorded trace level occurrence of natural and synthetic estrogenic hormones in the environment and even very low concentrations (10-100 ng/L) have potentially adverse consequences on the reproductive biology of vertebrates (Routledge et al., 1998; Schuh et al., 2011). Human and farm animal excretion of steroidal estrogens is a major source of estrogenic compounds which can potentially leach into surface and ground water (Finlay-Moore et al., 2000; Hanselman et al., 2004; Raman et al., 2004; Shore et al., 1993). High concentrations of estrogenic hormones and their partial breakdown products are often present in dairy manure and other wastewaters (Bradford et al., 2008; Hanselman et al., 2003; Hutchins et al., 2007; Kolodziej et al., 2004). A study on surface and well water in the vicinity of a cattle farm reported concentrations of estrone and 17α-estradiol up to 4.5 ng/L and 7.4 ng/L, respectively (Fine et al., 2003; Irwin et al., 2001). Municipal wastes were found to contain estrogens concentrations three to four times lower than that of wastewater originated from agricultural activities (Shore et al., 1993). Therefore, emerging pollutants such as the excretion of estrogenic hormones originating from agricultural activities can, even at very low concentrations, negatively impact the reproductive biology of aquatic vertebrates (Irwin et al., 2001; Jobling et al., 1998; Thorpe et al., 2003).

Antibiotics used for non-therapeutic functions for livestock animals such as cattle and swine accounts for 70% of the total annual antibiotics used volume in the USA (11,200 tons); of which, a large fraction is left unaltered in livestock excrement according to the Union of the Concerned Scientists (Karthikeyan & Meyer. 2006). Antimicrobials and antibiotics have been

abundantly reported in rivers, wastewater, animal waste, groundwater, and lakes according to many previous studies (Gilbertson et al., 1990; Gilbertson et al., 1995; Karthikeyan & Meyer. 2006; Meyer et al., 1999; Meyer et al., 2000). Multiple antibiotic residues were found at μg/L to mg/L levels from analyses of wastewater from CAFOs (Mackie et al., 2006; Meyer et al., 2000).

The evolution of antibiotic resistance as defense mechanisms is exacerbated by the abundance of residual antibiotics that are released into the environment which exert selective pressures on microbial ecosystems. Over the past few decades, evidence of antimicrobial chemical resistance has been prevalent. One noteworthy example includes the drastic increase from 39% to 97% of the proportion of Salmonella isolates exhibiting multiple drug and antibiotic resistance to ampicillin, streptomycin, streptomycin, tetracycline, and sulfonamides between 1980 to 1990 (Angulo. 1997; Lee et al., 1994). The emergence of tetracycline resistant genetic material in multiple locations near the swine housing at a CAFO, including groundwater 250 meters away from the lagoon and the manure lagoon serving that CAFO was reported by Chee-Sanford et al., (2001).

Irrigation, land application, overland run off, and leaching of animal waste during storage are major sources of CECs in animal waste which can enter the environment (Halling-Sorensen et al., 1998; Sarmah et al., 2006). Thus, surface and ground water contains residues of antibiotics and genetic material that encodes for antibiotic resistance (usually plasmids). From 1998 to 2009, antibiotic resistant infection treatment saw a surge in cost from $5 billion to $50 billion (Lederberg. 1998). Therefore, better understanding the fate, transport, and transformation of estrogenic hormones and antibiotics in animal wastes and the development of waste management processes which effectively lower the environmental discharge of these compounds is of high economic interest and priority.

1.5 THERMOCHEMICAL WASTE-TO-ENERGY PROCESSES TO MITIGATE CECS

Utilization of thermochemical bioenergy production processes which use elevated heat and pressure on manure is one promising solution to reducing the discharge of CECs from CAFOs and transforming organics into bioenergy resources. Of numerous processes, we will focus on two such approaches; catalytic hydrothermal gasification (CHG), which creates a methane-rich biogas product, and hydrothermal liquefaction (HTL), which produces a bio-crude oil product. Manure slurries with high water content and other wet feedstocks are most effectively utilized through these approaches. Because a valuable co-product can be produced while treating the waste, these processes are economically advantageous over numerous other waste treatment methods.

This research mainly focuses on the consequences of CHG operating variables on emerging contaminants which are derivatives of the mixed biomass harvested from the MABB. Although bioactive CECs in biomass can be completely deactivated by the thermochemical waste-to-energy procedure, other types of potential CECs may be generated (Pham et al., 2013). The CHG processing of the mixed biomass insures that bio-active compounds, including estrogenic hormones (E1, E2, E3, and EE2) and FF, can be destroyed effectively. The relative performance of utilizing different thermochemical waste-to-energy processes to remove CECs is important information that we have not much data on. CHG treatment would be a potentially important thermochemical process which we could effectively compare with the previous studies with HTL for CECs removal. CHG has demonstrated its efficacy in producing biogas consisting of methane, hydrogen, and carbon dioxide from various organic compounds. For example, Elliott and Sealock, (1996) subjected a Raney nickel catalyst (350°C, 21 MPa) under CHG of olive processing wash water to achieve 99.9% COD reduction. Similarly, a nylon manufacturing wastewater reported reduced COD concentrations from 1200 mg/L to 50-55 mg/L under similar conditions (Elliott. 1993). Using a Raney nickel catalyst or a Sn-modified Raney nickel catalyst reported degradations

of 90 - 100% of certain organic compounds such as phenol, aniline, tetrahydrofuran, toluene, and cyclohexanol (Li et al., 2008). However, common potentially problematic CECs in manure liquids have little research reported to date. Therefore, we plan to discover the potency of varying HTL and CHG operating conditions on the fate and identification of CECs and its breakdown products in this study. Through this, we can simultaneously present data supporting the process modeling of the transformation and transport of CECs in manure management systems and the most effective operating conditions to convert waste into bioenergy while largely eliminating the hazards associated with CECs present in animal manure. The data compounded thus far only covers a fraction of operating conditions and CECs which needs to be removed, demonstrating that there is room for optimization of CHG operating conditions to generate bioenergy and remove CECs, simultaneously. Thus, expanding this data set with more operating conditions and analyzing other CEC compounds would be ideal to present a stronger argument which can better support operation, rational design, and optimization of these procedures.

With this study, providing both environmental and bioenergy advantages is possible through expanding our previous work of investigating the removal of bioactive CECs present in the dilute LPAM utilizing similar integrated treatment methods. One possible approach would be to treat dilute LPAM with adsorbents as part of the wastewater treatment section. Regenerable adsorbents have proven to be useful in the destruction of various chemicals of emerging concern according to previous studies (Bolong et al., 2009; Petrovic et al., 2003; Rossner et al., 2009; Snyder et al., 2007). Both powdered (PAC) and granular activated carbon (GAC) are useful adsorbents which can simultaneously remove carbon while not leaving any harmful byproducts and be readily recovered, reformed, and reused as stated by Snyder et al., (2007). The adsorbed organics from biological removal can continuously provide *in-situ* regeneration of GAC adsorption

capacity in addition to GAC having been used simultaneously with microbial biodegradation methods (Craveiro De Sa & Malina. 1992; Speitel & Digiano. 1987; Vinitnantharat et al., 2001).

1.6 RESEARCH PURPOSES AND APPROACHES

Filtering CECs from animal manure and transforming them into valuable bioenergy products to prevent further pollution of the environment by developing a novel manure treatment system is the ultimate goal of this project. Investigating opportunities for the reuse of the aqueous fraction of manure is synonymous with reducing water pollution, which provides promising environmental benefits. The main objectives of this study are 1) Describing properties of the liquid portion of animal manure (LPAM) and the effects of CECs in LPAM through the application of analytical methods. 2) Demonstrating the ability and efficiency of constructing a feedstock source for bioenergy production with animal waste by extracting and concentrating CECs and other organics from LPAM to feed algal reactors. 3) Analyzing the fate of bioactive CECs subjected to hydrothermal bioenergy production systems. 4) Quantify the toxicity of wastewater after various treatment processes such as the effluent of MABB, HTL-WW, CHG-WW, and the organic mixture of LPAM in the manure treatment system. 5) Construct an efficient process model concerning the transport, fate, and transformation of CECs with process performance data. In conclusion, removal of most natural estrogenic hormones (<<10 ng/L) and typical antibiotics from manure is possible by subjecting wastewater to newly developed manure management processes including hydrothermal liquefaction (HTL), catalytic hydrothermal gasification (CHG), and the mixed algal bacterial bioreactors (MABB).

Defining the effects of CHG on bioactive contaminants in wastewater bioenergy processes which have previously not been characterized is the fundamental goal of this research effort. Specifically, we analyzed the efficiency in the removal of bioactive compounds from bio wastes

and the disruption of antibiotic resistant capacity or bacterial toxicity/CHO cell cytotoxicity of CHG operating conditions. Our hypothesis is that deactivation and degradation of antibiotics as well as a wide range of organic compounds is probable under the high pressure and elevated temperatures of CHG processes (400-550°C, 60 minutes, and Ru catalyst). As a result, human and livestock wastewaters that contain antibiotics and estrogenic hormones are commonly associated with ecosystem and health risks which could be alleviated, and water reuse potential can be augmented. Additionally, the creation of new antibiotics and antibiotic resistant therapy would potentially diminish in cost over the long-term. Also, the possibility of producing new toxic compounds in the original feedstock by the CHG process on bio wastes or algae is another relevant concern which needs to be addressed. Accessing the associated toxicity of the organic parts in CHG-WW and characterizing the chemical properties and makeup of CHG-WW was necessary to answer this concern. The current study describes a novel and important integrated wastewater treatment system and bioenergy production process. In conclusion, this study will focus on multiple national goals of augmenting cultivating biomass with better water quality, bioenergy production, and enhancing the potential for beneficial reuse and recycling of wastewaters.

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CHAPTER 2. LITERATURE REVIEW

2.1 OCCURRENCE OF CHEMICALS OF EMERGING CONCERN AND THEIR IMPACTS

2.1.1 OCCURRENCE OF NATURAL ESTROGENIC HORMONES FROM LIVESTOCK MANURE

In the aquatic environment, the increasing concentrations of pharmaceuticals and steroid hormones have been deemed unsafe for the environment (Boyd et al., 2003; Heberer. 2002; Kolpin et al., 2002; Snyder et al., 2003; Yu et al., 2011). The major sources of pharmaceuticals, personal care products (PPCPs), and naturally arising hormones to nearby watersheds via wastewater effluent are sewage treatment plants (STPs) and CAFOs (Benotti & Brownawell. 2007; Kolpin et al., 2002; Snyder et al., 2003; Zheng et al., 2008). STPs and livestock manure disposal units commonly discharge natural estrogens, such E1, E2, E3, and EE2 into the environment (Combalbert et al., 2012; Raman et al., 2004; Shi et al., 2004; Ternes et al., 1999b). Even at nanogram per liter concentrations, E1 and E2 can cause feminization of male fish in aquatic environments worldwide (Cao et al., 2010; Shi et al., 2013; Sumpter & Jobling. 1995).

A large fraction of estrogens in the environment is produced by animals, especially livestock in CAFOs. The species, sex, age, circadian cycle, and reproductive state of the animal are variables which determine the type and quantity of excreted estrogens (Combalbert & Hernandez-Raquet. 2010; Hanselman et al., 2003). Urine and feces from livestock typically contain estrogens which are biologically active free compounds or inactive compounds conjugated with glucuronide and/or sulfate groups (Hutchins et al., 2007; Panter et al., 1999). Conjugated estrogens are more soluble and easier to excrete because estrogen compounds are normally conjugated to sulfate or glucuronide forms by substituting the hydroxyl groups in the livers of livestock (Combalbert & Hernandez-Raquet. 2010). Estrogenically inactive forms can be

hydrolyzed to active free estrogens via glucuronidase and sulfatase enzymes by intestinal and fecal microorganisms such as *Eschericia coli* (Coleman et al., 2004; Ternes et al., 1999a). However, sulfate conjugates were found in both agriculture watershed receiving livestock manure and sewage treatment plants (Dutta & Dawid. 2010; Kumar & Mohan. 2011). Therefore, investigating the occurrence and fate of conjugated estrogens present in both the livestock excreta and manure treatment facilities is necessary to quantify the occurrence of total estrogens. However, only free estrogens are analyzed in this study because conjugated estrogens do not exhibit estrogenic activity before hydrolysis and conjugated estrogens are resistant to hydrolysis.

2.1.2 CONSUMPTION OF ANTIBIOTICS AND ESTROGENS IN THE AGRICULTURAL FIELD

Since the early 1950s, disease prevention, growth promotion and feed efficiency enhancement in food animals was induced by antimicrobial agents and hormones. Animals, with the help of antibiotics, can better absorb animal feed and expedite growth. In addition, animal susceptibility to crowded living situations and poor hygiene in intensive animal agriculture can be minimized by supplementing antibiotics in animal feed (EMS, 2000). 80% of the total quantity of antibiotics (36 million pounds) produced in the U.S. are used in food animal production according to a recent report by the U.S. Food and Drug Administration produced in 2009. Rather than treatment of infections, 90% of antibiotics used on food animals are for growth promotion. Since the 1950s, the recommended levels of antibiotics for feeds have grown 10 to 20-fold whereas, initially, concentrations were limited to approximately 5-10 ppm (Khachatourians. 1998). Previous studies found that enhancement of animal growth and immunization required sub-therapeutic levels of antibiotics in feed (3-220 g/Mg feed) (Feinman et al., 1978; Gavalchin & Katz. 1994). According to Flanders and Gillespie, (2016), the growth of swine and poultry was improved following the consumption of antibiotics such as bacitracin, chlortetracycline, erythromycin,

lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tyrosine or virginiamycin at concentrations of between 1 and 500 g of antibiotics per ton of livestock feed. Despite low antibiotic concentrations being able to place selective pressures on bacteria, animals are administered antibiotic concentrations much higher than the recommended dose. For example, up to 25% of feeds contained antibiotic concentrations higher than the recommended levels in an examination of 3,328 feeds conducted by the US National Swine Survey (Dewey et al., 1997).

The agricultural consumption of antibiotics far outnumbers the human consumption of antibiotics. An estimated 100-1000 times more antibiotics are used on animals versus humans, annually (Feinman. 1998; Levy. 1998; Witte. 1998). Concerns of antibiotic resistant genes in animal production and potential risks to human health have been voiced over the extensive use of antibiotics in agriculture. Over recent decades, an increasing number of people associated with agricultural use of antibiotics have reported resistant infections. Between 1973 and 2011, 34 out of 38 foodborne outbreaks had antibiotic resistant patterns according to the Center for Science in the Public Interest (CSPI) (CSPI. 2012). Nine of 15 different antibiotics which were found to be ineffective on the bacteria that caused the outbreaks are classified by the World Health Organization (WHO) as “critically important” to medicine. Over 20,000 people were infected in these outbreaks and 27 of the 3108 hospitalized died. Approximately two million Americans are infected and 14,000 Americans die annually as a result of antibiotic resistant pathogens according to a report by the WHO (WHO. 1999). Consequentially, the U.S. annually invests an estimated $100-200 million to treat all drug resistant infections (Cassell. 1997). Therefore, the risk of resistant infections in humans is directly correlated to the increased use of antimicrobial agents. As a result, control measures to negate the dissemination of antibiotics and antibiotic resistant genes stemming from the 28 agricultural uses of antibiotics is of critically important.

Since the 1950s, animal food production has used steroid hormone drugs, including natural estrogen, progesterone, testosterone, and their synthetic versions to artificially stimulate animal growth. Animal manure and urine contained a significant portion of these compounds. In poultry or livestock manure, elevated concentrations of E1 and E2 had been detected (Nichols et al., 1997; Peterson et al., 2000; Short & Colborn. 1999; Ternes et al., 1999b). An estimated quantity of a couple milligrams per animal of excreted hormones is produced daily (Hanselman et al., 2003; Lange et al., 2002). Estimated annual excretion of estrogens from livestock animals such as cattle, pigs, sheep, and chickens were 39 tons in the European Union and 41 tons in the United States (Lange et al., 2002). Another environmental concern discusses the use of estrogenic compounds as agricultural fertilizer, which potentially exhibit endocrine disrupting effects as water resources can be contaminated and even low estrogen concentrations can cause adverse ecological effects (Irwin et al., 2001; Jobling et al., 1998; Thorpe et al., 2003). For example, vitellogenin production in male organisms is induced by E1 and E2 at concentrations as low as 1 ng/L (Purdom et al., 1994). Also, production of synergistic estrogenicity responses may be possible in mixtures of estrogenic compounds (Daughton & Ternes. 1999; Sumpter & Jobling. 1995). Therefore, the fate and transport of manure born estrogenic compounds in the treatment process is of high importance.

2.2 FATE AND TRANSPORT OF CHEMICALS OF EMERGING CONCERN

2.2.1 LAND APPLICATION OF LIQUID LIVESTOCK WASTEWATER

Surface runoff or land-applied composts could contain discharge of estrogens from livestock waste which can seep into the aquatic environment. Manure applied agricultural fields poses a potential threat to surrounding groundwater and surface water as the leaching and migration of estrogens expands the range of contamination (Belfroid et al., 1999; Dutta & Dawid. 2010; Snow et al., 2015). E1, one of the most commonly observed estrogens in manure, was

detected in runoff (Dutta & Dawid. 2010). Dutta et al. 2010). However, no reports on the fate and occurrence of conjugated estrogens in the compost exist. Therefore, it is of interest to determine the fate and excretion of conjugated estrogens during composting. Also, despite restrictions of animal feed additives by the Chinese Ministry of Agriculture (MOA The Ministry of Agriculture of the People's Republic of China 2013), wastewater effluents from CAFO facilities and nearby receiving rivers in China were discovered to contain synthetic estrogens, such as EE2 which serve human contraception purposes, in addition to natural estrogens (Liu et al., 2012; Zhou et al., 2010).

2.2.2 TRANSPORT OF BIOACTIVE CECS IN THE ENVIRONMENT

Since animal intestines are poor absorbers of antibiotics, as much as 30–90% of the parent compound can be detected in feces or urine (Alcock et al., 1999; Elmund et al., 1971). Despite treatment and storage of wastes, a large volume of these drugs persist and exert selective pressures on microbial communities after being discharged into the environment. Subsequently, the development of antibiotic resistances as defense mechanisms is induced. Approximately 1.32 billion Mg of manure is produced annually according to the United States Department of Agricultural (USDA). An increase in antimicrobial resistance in the environment is inevitable due to natural selection as antibiotic toxicity to soil microbial communities increase due to the presence and persistence of antibiotics in large quantities of manure, presenting a significant environmental concern. The dominant pathway for the discharge of estrogenic compounds and antibiotics into the terrestrial environment is through the land application of manure. Both human and environmental health is impacted negatively as antibiotics in manure can seep into ground water, accumulate in the soil, or leach into surface water (Jongbloed & Lenis. 1998). The permeation of estrogenic compound residuals in animal manure into surface and ground water via runoff is highly probable. (Lange et al., 2002). Manure containing wastewaters often report high concentrations of

estrogenic hormones and their partial breakdown products (Bradford et al., 2008; Hanselman et al., 2003; Hutchins et al., 2007; Kolodziej et al., 2004).

The presence of estrogenic compounds and antibiotics in animal manure and soil is commonly reported. For example, manure wastes reportedly contain antimicrobial classes such as tetracyclines, sulfonamides, β-lactams, macrolides, and ionophores (Kumar et al., 2012; Meyer et al., 2000). Antibiotics detected in soil fertilized with pig manure is represented. The soil environment contained a considerably lower concentration of detected antibiotics than in manure despite varying significantly. The data suggests that attributes of soil environments and antibiotic chemical characteristics determines the persistence of antibiotics in the soil environment.

Numerous studies have reported the occurrence of antibiotics in the aquatic environment (Ashton et al., 2004; Golet et al., 2001; Hirsch et al., 1999; Kolpin et al., 2002; Rice et al., 2001; Sacher et al., 2001; Teuber et al., 1999). For instance, surface water samples in Northwest Germany contained a wide range of antibiotics, including sulphonamides, macrolides and lincosamides (Christian et al., 2003). Lagoons and groundwater under two swine production facilities harbored tetracycline resistant bacteria (Chee-Sanford et al., 2001). The groundwater impacted by swine production facilities also found tetracycline residues and tetracycline resistance genes (Mackie et al., 2006).

2.3 ALGAL BIOREACTOR FOR BIOMASS PRODUCTION AND WASTEWATER TREATMENT

2.3.1 NUTRIENT REMOVAL

Typically, 45-60% of microalgae dry weight is composed of high amounts of nitrogen and phosphorous for nucleic acids, phospholipids, and proteins (Munoz & Guieysse. 2006). Between 1% and 14% of algal dry weight composition is nitrogen and between 0.05% and 3.3% for

phosphorus (Richmond 2004). NH_3 stripping or phosphorus precipitation because of a pH increase during carbon dioxide fixation and photosynthesis can further optimize nutrient removal. Several algal species such as *Botryococcus braunii*, *Chlamydomonas*, *Scenedesmus*, and *Chlorella* have reported useful nutrient removal. Secondary effluents in both batch and continuous experiments successfully cultivated *Botryococcus braunii*, removing up to 99% of nitrates and 93% of phosphates (Sawayama et al., 1994; Sawayama et al., 1995). According to Tam and Wong, (1996), *Chlorella vulgaris* in wastewater reported a removal efficiency inversely related to the original nitrogen concentration. At initial nitrogen concentrations > 20mg/L, 100% nitrogen removal was achieved. However, at higher concentrations, absolute nitrogen removal was impossible but partial nitrogen removal was still possible. At 40-80 mg/L, 95% nitrogen removal was observed and at concentrations above 80 mg/L, 50% nitrogen removal was observed.

High efficiency in nutrient removal was also achieved in mixed-cultures of algae. 40-98% nitrogen removal and 40-90% phosphorous removal in swine and dairy manure was reported by utilizing the algae turf scrubber (ATS) (Kebede-Westhead et al., 2003; Pizarro-Cerda & Cossart. 2006; Pizarro-Cerda et al., 2012).

2.3.2 SYMBIOTIC RELATIONSHIP BETWEEN ALGAE AND BACTERIA

Traditionally, a conventional secondary treatment like activated sludge removes most organics before microalgae is used for N and P removal (Lavoie & Delanoue. 1987). However, significant organic removal can be possible by algae, according to some recent studies (Dilek et al., 1999; Hodaifa et al., 2008; Kamjunke et al., 2008).

Organics can be consumed by algae, such as heterotrophic bacteria, but the assimilation of organics is a more complex procedure. There are four classifications of algae, which are autotrophic algae, heterotrophic algae, mixotrophic algae, and photoheterotrophic algae (A. H.

Neilson. 1974). Sustained growth and cell division in the dark are traits of heterotrophy in algae, which is exclusively an occurrence by aerobic dissimilation. These algae perform substrate respiration similar to that of heterotrophic bacteria in which oxygen is consumed and carbon dioxide is expelled. Most heterotrophic algae also grow photoheterotrophically, except for a few colorless algae spices, e.g., *Prototheca zopfii*, which are obligate heterotrophs. Algae with impaired abilities to assimilate carbon dioxide in the presence of light utilize mixotrophy. Thus, even with light, mixotrophic algae demands a supply of organic carbon for growth. Generally, smaller amounts of carbon dioxide are simultaneously assimilated than that required for phototrophic growth. Many algae perform photoheterotrophy (photoassimilation) which requires a steady source of light. Therefore, algae are unable to heterotrophically grow in the dark, but can produce cellular material, such as lipids, by consuming certain organic compounds in the light. *Chlamydonomas*, for example, can also assimilate lipids from exogenous acetate. Some algae are even adept at assimilating lipids from long-chain fatty acids without prior degradation (A. H. Neilson. 1974). Although possible under certain laboratory conditions, algae which perform organic material assimilation are unable to effectively compete with other, rapidly growing heterotrophic organisms because of low substrate affinities (A. H. Neilson. 1974).

2.3.3 ALGAL BIOREACTOR WITH ACTIVATED CARBON

Aerobic degradation of numerous organic contaminants illustrated the symbiotic relationship between algae and bacteria. Heterotrophic bacteria for mineralizing organic pollutants requires O_2 which can be produced by algae and the subsequent CO_2 released from bacterial respiration would fuel photosynthesis in algae respiration, as shown in Figure 2.1 (Munoz & Guieysse. 2006). Mixed algal-bacterial wastewater treatment systems are becoming increasing popular for two main reasons. First, the economic advantages of mechanical aeration through

photosynthetic aeration by producing oxygen from algae accounts for more than half of the total energy consumption for common aerobic wastewater treatments (Metcalf et al., 2003). Second, biofuel feedstocks can be made from algal biomass (Gouveia & Oliveira. 2009; Mata et al., 2010; Rodolfi et al., 2009).

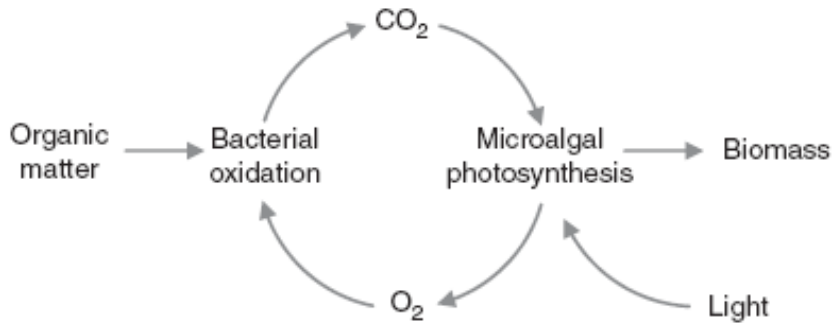

Figure 2.1 Principle of photosynthetic aeration in organic removal process (Munoz & Guieysse. 2006).

However, achieving acceptable levels of organic and nutrient removal can be challenging in combining an optimal mixture of algae and bacteria to treat wastewater. This is due to the fact that various organic pollutants and heavy metals inhibit algal growth according to Munoz and Guieysse, (2006), which are common occurrences in numerous wastewater streams. Additionally, the decreasing source of light as increasing bacteria growth negatively affects turbidity determines the growth relationship between algae and bacteria. Furthermore, heterotrophic algae generally grow slower than heterotrophic bacteria as reported by Kamjunke et al., (2008) and can produce a competitive pressure. Finally, algal assimilation of pollutants and nutrients is heavily dependent on environmental conditions such as sunlight and temperature.

Although nutrient and organic contaminants are typically removed by the algal bioreactor treatment, bacteria and algae are unable to biodegrade certain recalcitrant organic compounds.

Therefore, to completely remove all remaining compounds, biological activated carbon, membrane biological reactors, or other physical removal process are necessary. According to previous studies, various organic contaminants of concern are removable by employing GAC (Aksu. 2005; Tryba et al., 2003). Activated carbon adsorption and ozonation on a bench scale has been evaluated in previous studies for the treatment of petrochemical wastewater (Farooq & Misbahuddin. 1991; Kunz & Giannelli. 1976). Between 51-73% of total organic carbon (TOC) is removed by activated carbon from wastewater, according to batch adsorption studies. A reduction of 32.5% and 40% in TOC and COD, respectively, is possible through ozonation. Up to 81% of TOC was removed by the combined ozone and adsorption treatment (Farooq & Misbahuddin. 1991).

Thus, oxygen containing compounds, such as carboxylic acids, can be removed effectively by activated carbon, as demonstrated in previous studies alcohols and ketones (Aizpuru et al., 2003; Brasquet et al., 1999; Daifullah & Girgis. 1998; How & Morr. 1982; Vazquez et al., 2007; Ward & Getzen. 1970). Cyclic hydrocarbons and alkanes are similarly removed by activated carbon (Diaz et al., 2004; Malek & Farooq. 1996). More importantly, no harmful by-products are produced by the removal of these compounds and activated carbon can easily be recovered, regenerated, and reused. Therefore, a proper balance of algae and bacteria must be carefully designed to produce an optimal wastewater treatment system and GAC could be used to remove most of the residual contaminants in the wastewater.

2.4 HYDROTHERMAL PROCESSES OF BIOMASS FOR BIOENERGY PRODUCTION

2.4.1 HYDROTHERMAL LIQUEFACTION

Numerous studies have investigated the dependency of biocrude oil yield by HTL on the reaction time (Qu et al., 2003; Su et al., 2004; Xu & Lancaster. 2008; Yan et al., 1999). The reaction time is measured as the total time during the liquefaction reaction after having reached a designated

reaction temperature. Both the overall conversion efficiency of biomass and the distribution of HTL final products are affected by the reaction time. Generally, the rate of biomass decomposition and hydrolysis under supercritical conditions is relatively fast and a short reaction time (<60 minutes) can convert biomass into biocrude oil (Sasaki et al., 2003). Shorter residence times are usually preferred during hydrothermal biomass liquefaction. Additionally, different biomass feedstock has distinct optimal reaction times.

In most cases, increasing the reaction time generates higher biocrude oil yields. However, there still exists a ceiling after which biocrude oil yield decreases when the reaction time is increased (Boocock & Sherman. 1985; Su et al., 2004; Xu & Lancaster. 2008; Yan et al., 1999). (Boocock & Sherman. 1985). reported suppressed biocrude oil yields for longer residence times excluding biomass with very low water contents (Boocock & Sherman. 1985). Jena et al., (2011) similarly reported a decrease in biocrude oil yield after increasing reaction time during the liquefaction of *Spirulina* due to the conversion of lighter hydrocarbon compounds found in the bio-crude oil into gaseous products. For longer residence times on liquid yields, Yan et al., (1999) reported a negligible increase in liquid yields. Direct liquefaction of *Cunninghamia lanceolate* reported lower oil yields for longer reaction times (Qu et al., 2003; Sugano et al., 2008).

In fact, the reaction time also influences how prevalent the effect of reaction temperature on oil yield is. Karagoz et al., (2004) reported that liquid oil yield and conversion of sawdust was favored when reaction time was increased at low reaction temperatures (<150°C). However, the overall conversion of biomass and gas yield were increased for the biomass liquefaction at higher temperature (250–280°C). The effect of residence time on the liquefaction can be explained by many different reasons. For example, longer reaction time induces the dehydration of formic acid/acetic acid intermediates and carbohydrates which results in hydrolysis reactions that may account for the formation of more bio-crude oil, explaining why prolonged reaction time could

lead to an initial increase in bio-crude yield. Further prolonged reaction time resulted in the secondary and tertiary reactions in hydrothermal medium after biomass conversion reached its apex. Heavy intermediates can be converted into liquids, gases, or residues species which subsequently results in lower oil yields. Reaction time was also an important factor in determining the chemical composition and HTL product distribution of biocrude oil. Increasing the reaction time (30–120 minutes) enhanced gases yields, lowered water soluble fraction yields, and left no significant changes to solids residues (Jena et al., 2011a). The composition of the final products of HTL can be affected by variations in reaction time. For both low (180°C) and high (250°C) temperatures, the composition of oil products was different for long and shorter reaction time according to Karagoz et al., (2004). They found that 5-(hydroxymethyl)-2-furan carboxaldehyde, 4-hydroxy-3-methoxybenzoic acid, 4-hydroxy-3-methoxybenzeneacetic acid, and bis(2-ethylyhexyl) phthalate were observed in the biocrude oil at temperature of 180°C paired with a reaction time of 60 minutes. However, at reaction time of 15 minutes at the same reaction temperature, these compounds were not observed. A similar experiment with reaction variables of 280°C and 15 minutes observed the presence of vanillin, phenol, 2,4-dihydroxybenzaldehyde, and others in biocrude oil but not when the reaction time was for 60 minutes (Karagoz et al., 2004). In general, for long residence time, the oil yield decreases while gas yield and biomass conversion continuously increase until saturation (Su et al., 2004).

2.4.2 CATALYTIC HYDROTHERMAL GASIFICATION

Pyrolysis and/or partial oxidation are well-known methods in the gasification of biomass by thermal methods, producing a synthetic or fuel gas, composed of hydrogen and carbon oxides. Typically, a dry biomass feedstock with <10 wt % moisture is used. However, a large portion of

the biomass resource consists of high level moisture biomass, some containing 50 wt % and others consisting wet biomass or water slurries biomass at 85 wt % moisture or higher.

Hydrothermal gasification is operable over a range of temperatures and pressures. Supercritical water was considered as an important operating medium and the overriding parameter in earlier research efforts. However, further investigation suggests that subcritical water is effective on gasification when performed in-situ with active catalysts. Heat recovery is particularly important in efficient operation. The only major input is biomass/water slurry and after catalytic treatment, a simple separation of fuel water and gas is required. A recent publication updating the using conditions on both sides of the critical water point demonstrated an interest in the concept. Similarly, Kruse et al., (2000) provides a companion review on biomass hydrothermal gasification without the use of heterogeneous metal catalysts. Several significant effects arise as a result of the temperature used for the hydrothermal gasification of biomass. Osada et al., (2006) identified three temperature zones for hydrothermal gasification in their review. Region I (500–700 °C supercritical water) is reserved for resolving of biomass and avoiding char formation by using an activated carbon catalyst. Also, an alkali catalyst facilitates the water-gas shift reaction. In region II (374–500 °C, supercritical water), hydrolyzation of biomass and gasification was facilitated by metal catalysts. Finally, slow hydrolysis of biomass and gas formation requires catalysts in region III (below 374 °C, subcritical water).

A potential way to transform wet biomass to a fuel rich gas consisting of H_2 and/or CH_4 is through the gasification of microalgae in near or above critical water (Tc = 647 K, and Pc = 22.1 MPa). A dry feedstock is not required for this process of hydrothermal gasification; therefore, processing aquatic biomass is not an issue. Also, because water is both a reactant and reaction medium, hydrothermal gasification leads to low tar and char formation while producing high hydrogen yields. The literature suggests that hydrothermal gasification is a valuable procedure. A

supply of H_2 for hydrotreating or catalytic upgrading crude algal biocrude oil is required in an algal biorefinery. In addition, CH_4 from hydrothermal gasification can be used to provide process heat as fuel gas. Thus, an integrated algal biorefinery can operate solely on renewable energy resources through the gasification of algae. Unless under high temperatures (~600 °C), uncatalyzed hydrothermal gasification of microalgae produces low gasification efficiency. Lower temperature gasification also lowers the operating cost. Therefore, discovering a way to gasify microalgae with CECs at lower temperatures has a distinct economic incentive.

One such approach is catalyzed gasification. Ruthenium catalyst is an efficient catalyst commonly used for hydrothermal gasification. Sato et al., (2011) discovered that Ruthenium can efficiently decompose certain compounds observed from microalgae gasification in supercritical water (SCW), including alkylphenols, a class of ring-containing products, at 400 °C. Ruthenium has also been used for the gasification of compounds, including cellulose, lignin, glycerol, glucose, and the others in biomass. However, for microalgae, only three previous accounts of catalyzed hydrothermal gasification utilized Ru catalyst to the best of our knowledge. Chakinala et al., (2010) reported no gas compositions at lower temperatures and a single instance for gasification utilizing Ru at 600 °C, which aligns with the interest of this work. Haiduc et al., (2009) reported three experiments using Ru, all near 60 minutes of reaction times. Stucki et al., (2009) worked extensively on the Ru-catalyzed experimental gasification of algae, but the focus relied more on comparing different catalysts and supports. There has yet to be a thorough study on the effects of process variables in Ru-catalyzed gasification in water above the critical temperature.

2.5 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF WASTEWATER

2.5.1 ANTIBIOTIC RESISTANCE CAPACITY

The use of antibiotics on livestock has resulted in the appearance of bacterial resistance to them and the isolation of the mutant genes (Alexander et al., 2009; Aslam et al., 2010; Binh et al., 2008; Cessna et al., 2011; Heuer et al., 2009; Peak et al., 2007). Indeed, swine manure is the repository of many such bacteria (Binh et al., 2008; Heuer et al., 2009). In a study of swine in Northern Ireland, Watabe et al., (2003) found that 57.7% of the Salmonella in the animal waste were resistant to at least two antibiotics, 34.6% to three and the rest to four. Chee-Sanford et al., (2001) detected antibiotic-resistant genes (ARGs) in bacteria taken from an animal feeding operation and also in the groundwater 250 meters downstream from the location. Zahn et al., (2001) compared livestock fed antibiotics to those who weren't and resistance indexes were 3-fold higher in the first group. Resistant bacteria have been found in wastewater samples from hospitals, agriculture and aquaculture operations, wastewater treatment facilities, and tap water; subsequently, their proliferation threatens the environment and human health according to Ghosh and LaPara, (2007) and has escalated to significantly hindering the effective treatment of human illnesses (Institute of Medicine, 1998).

Therefore, both antibiotics and resistant bacteria should be closely monitored and, in serious cases, restricted in commercial effluents (Pruden et al., 2006). Also, discharging steroids in the environment can be considered malicious. Their sources and journeys need to be traced and understood. A significant number of studies have investigated this particular topic (Gulkowska et al., 2008; Snyder. 2008; Ternes et al., 2002; Ternes et al., 2003; Zorita et al., 2009). To date, the treatment methods have not reached an acceptable standard (Janssen et al., 1997; Suidan et al., 2005; Sumpter & Johnson. 2005). These efforts must be significantly augmented to develop cost-

effective treatment methods that preserve the purity of the environment from estrogenic compounds and antibiotics effective for treating diseases.

2.5.2 ESTROGENIC ACTIVITY

Estimation of total estrogenic activity is possible through In vitro bioassays which can be a rapid, sensitive, inexpensive, and integrative screening procedure by analyzing all compounds in mixtures which act through the same mode of action (Hilscherova et al., 2000). Simple compounds and complex mixtures can be measured for estrogenic activity with several in vitro bioassays. The natural processes of endocrine systems can be interfered by chemicals in several ways such as affecting the synthesis or metabolism of natural hormones, binding to hormone receptors, and/or disrupting hormone receptor synthesis or metabolism (Katzenellenbogen and Muthyala, 2003; Zacharewski, 1997).

Estrogenicity detection is most frequently performed by in vitro transactivation assays (Kinnberg, 2003) which determine the capacity for samples to stimulate estrogen receptors and upregulate subsequent reporter gene expression (and in vitro estrogenicity assays). Additionally, tiered monitoring of environmental waters is currently being evaluated by in vitro estrogenicity assays (Leusch et al., 2010). Comparing estrogenic activity of environmental samples determined by different in vitro assays demonstrate that the assays are effective for environmental monitoring (Leusch et al., 2010; Murk et al., 2002).

Genetically engineered yeast, fish, or mammalians cells are normally subjected to transfection with an estrogen-responsive element (ERE) DNA sequence connected to a reporter gene when conducting reporter gene assays (Zacharewski, 1997; Kinnberg, 2003). After an agonist binds to the receptor, a series of molecular events results in the receptor binding to the ERE and activating the gene expression process (Figure 2.2). The reporter gene then generates a product

that can be measured appropriately (galactosidase or luciferase genes represent most reporter genes, with protein/enzymatic products easily quantified by spectrophotometry and luminometry). Vertebrate assays are typically performed with fish (Ackermann et al., 2002; Rutishauser et al., 2004) or mammalian cells (Legler et al., 1999; Vinggaard et al., 1999; Balaguer et al., 2000; Wilson et al., 2004) of which the luciferase gene is usually transfected with an ERE. Simple yeast-based assays (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998; Garcia-Reyero et al., 2001) reportedly transfect yeast cells with a galactosidase reporter gene linked mammalian ERE. An expression plasmid containing a mammalian ER is also introduced as yeast do not possess an endogenous ER. Finally, yeast Gal4 DNA binding domains and chimeric receptors with a mammalian ligand binding domain are utilized by chimeric yeast reporter gene assays which harvest the natural yeast genetic machinery (Nishikawa et al., 1999). Field research demonstrates the persistence of estrogenic properties in soils amended with biosolids. Soil from plots in Australia were measured for the YES bioassay response described above (Langdon et al., 2012). Even at low concentrations, estrogenic activity persisted in the treat soils (with control plots exhibiting no measurable estrogenic response) even after 4 months of application.

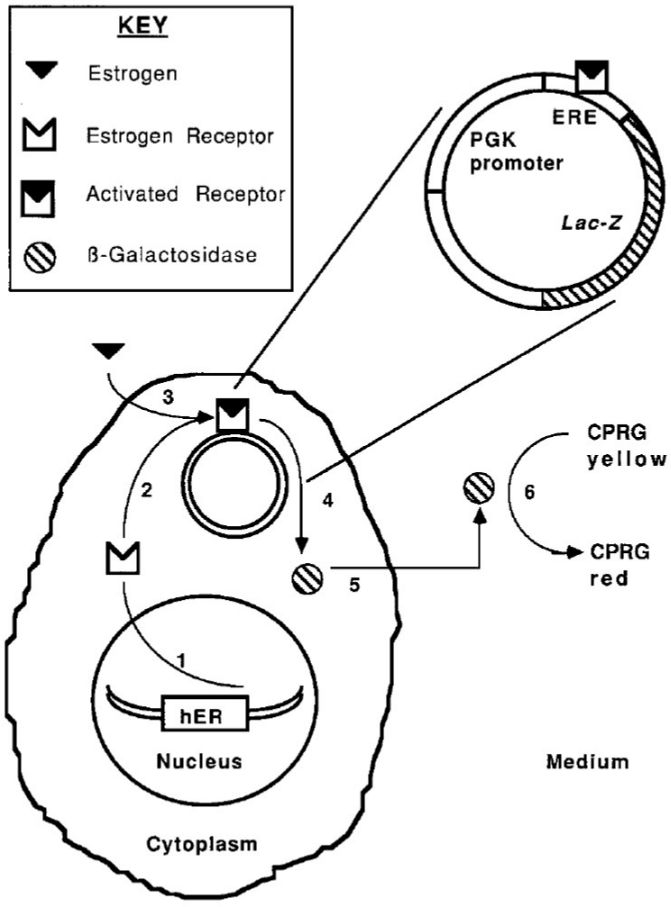

Figure 2.2 Principle of the YES Assay (Routledge and Sumpter, 1996)

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CHAPTER 3. CHARACTERIZATION OF BIOACTIVE CONTAMINANTS FROM THE LIQUID PORTION OF ANIMAL MANURE AND THEIR IMPACTS

3.1 INTRODUCTION

Naturally occurring estrogens and antibiotics in animal waste can impact the environment negatively primarily through the disruption of the endocrine system in animals, and humans (Khanal et al., 2006). Excretion of steroidal estrogens from humans and farm animals is the major source of estrogenic compounds in the environment and can potentially contaminate surface and ground water (Finlay-Moore et al., 2000; Hanselman et al., 2004; Raman et al., 2004; Shore et al., 1993). As shown in Figure 3.1, the bioactive contaminants from animal manures can be discharged to the environment via irrigation and drainage and have the potential to increase antibiotic resistance in pathogenic microorganisms or disrupt normal endocrine functions.

Significant concentrations of estrogenic hormones and their partial breakdown products are commonly reported in wastewaters containing manure (Bradford et al., 2008; Hanselman et al., 2003; Hutchins et al., 2007; Kolodziej et al., 2004). For example, the concentrations of 17-estradiol and estrone in surface water and well water near cattle farms have been reported at 7.4ng/L and 4.5ng/L, respectively (Fine et al., 2003; Irwin et al., 2001). Understanding the fate, transport, and transformation of these bioactive compounds in livestock systems and developing management practices that cost-effectively mitigate the associated risks are particularly important. To enhance our understanding of these issues, it is necessary to develop or demonstrate a set of analytical methods that can be used in the complex matrix of LPAM to evaluate the performance of proposed treatments. In previous work on the bioactive chemicals of emerging concern (CECs)

in manure and hydrothermal liquefaction (HTL) matrices, there were difficulties in analysis caused by the significant background noise commonly associated with complex matrices containing significant organic content. Since chemically identifying all individual CECs in the liquid portion of animal manure (LPAM) is quite difficult and perhaps impractical, an in vitro toxicological approach was used to provide a more comprehensive analytical method to evaluate the removal of undesirable chemical compounds in LPAM. This section describes a suite of analytical methods that were used to characterize LPAM before and after each treatment process applied to animal manure samples.

The objectives of this section are to 1) Investigate the occurrence of natural estrogenic hormones from a swine farming system to evaluate the production rate of hormones. 2) Evaluate the fate and transport of CECs from manure during sedimentation and filtration processes to prepare sample of LPAM used throughout this study. 3) Characterize the LPAM chemically and biologically including water quality analysis and acute toxicity assays.

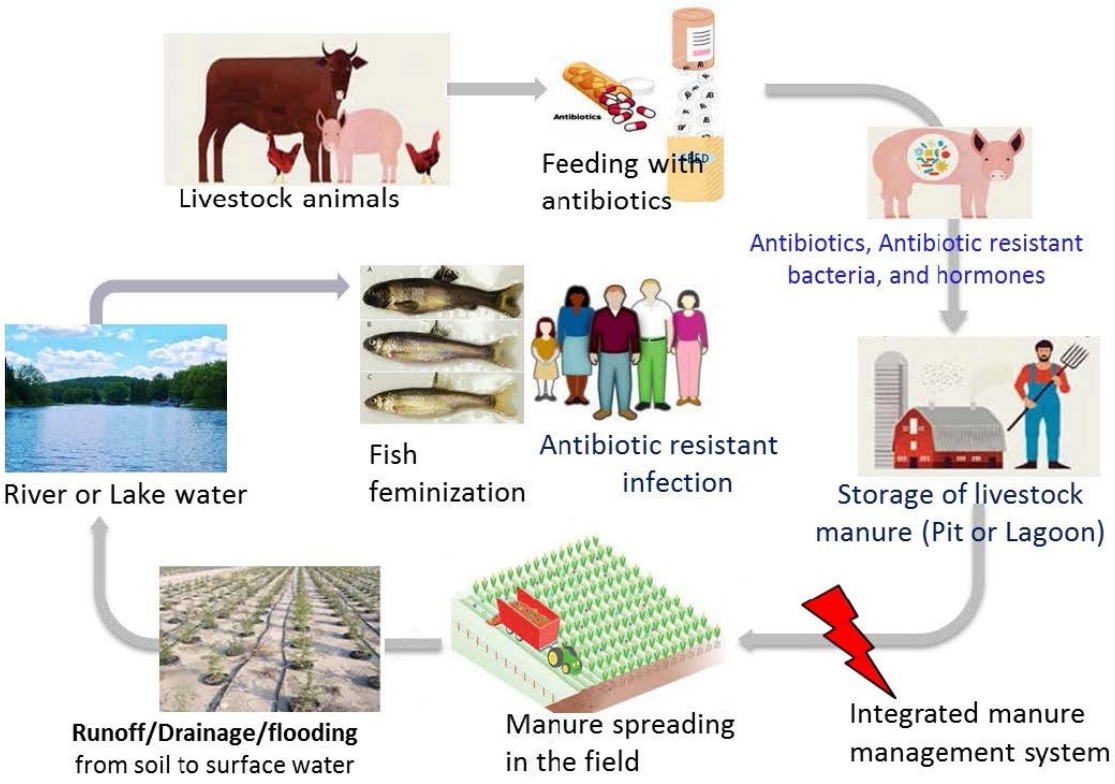


Figure 3.1 Pathways for transport of bioactive compounds from manure that can affect the health of humans and ecosystems.

(Adapted from: https://blog.epa.gov/blog/2014/06/synthetic-female-hormones-in-sewage-are-toxic-to-male-fish-over-generations/)

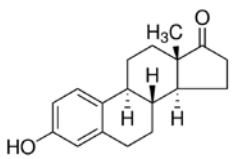
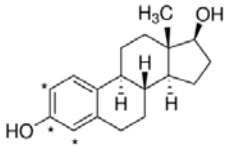
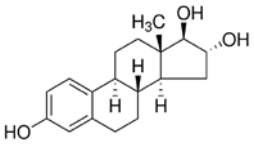
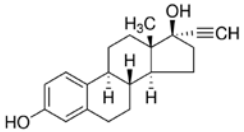
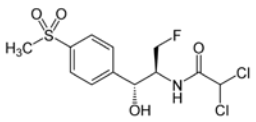
3.2 MATERIAL AND METHODS

3.2.1 CHEMICALS AND REAGENTS

The steroid hormones estrone (E1, ≥99%), 17β – estradiol (E2, ≥98%), estriol (E3, ≥97%), and 17α – estradiol (EE2, ≥98%) were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI) and used as hormone standards. The stock solutions of E1, E2, E3, and EE2 at 2g/L were prepared in pure methanol, and Table 3.1 showed the properties of selected estrogenic hormones and FF. Solid phase extraction (SPE) columns such as the Supelclean Envi-Carb (Capacity: 500 mg/6mL, No. 57094) and Supelclean LC-Florisil (1 g/6mL, No. 57057) were purchased from

Supelco (Bellefonte, PA, USA). Distilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade organic solvents such as n-hexane, acetone, acetonitrile, methanol, or dichloromethane were purchased from Fisher Scientific (Fair Lawn, NJ). The XenoScreen XL YES assay kit was purchased from Xenometrix AG (Allschwil, Switzerland). β-glucuronidase (No. G0876) and sulfatase (No. S9751) were purchased from Sigma-Aldrich.

Table 3.1 Properties of selected estrogenic hormones and antibiotic (Hanselman et al., 2003; Zhiquang et al., 2004, A.K. Sarmah et al., 2006)

Properties	**Estrone**	**17β-estradiol**	**Estriol**	**17α-estradiol**	**Florfenicol**
Used abbreviation	E1	E2	E3	EE2	FF
Class	Steroid	Steroid	Steroid	Steroid	Antimicrobial
Cas registry number	53-16-7	50-28-2	50-27-1	57-63-6	73231-34-2
Molecular weight (g/mol)	270.4	272.3	288.4	296.4	358.21
Vapor pressure (Pa)	3×10^{-8}	3×10^{-8}	9×10^{-13}	6×10^{-9}	Negligible
Water solubility (20°C, ppm)	0.8 - 12.4	3.9 - 13.3	3.2 - 13.3	4.8	over 400 at pH > 5.5
pK_a	10.3 - 10.8	10.5 - 10.7	10.4	10.21	9.03
log K_{ow}	3.1 - 4.0	3.1 - 4.0	2.6 - 2.8	3.67, 4.15	2.36
Molecular formula	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_3$	$C_{20}H_{24}O_2$	$C_{12}H_{14}Cl_2FNO_4S$
Structure					

3.2.2 SYSTEM DESIGN AND SAMPLING

Swine manure samples were collected twelve times over fourteen months to characterize variations in water quality parameters and CECs of the liquid portion of swine manure (LPAM). Samples were taken from multiple locations within the swine farm including from gestation, farrowing, and finishing operations at the Swine Research Center (SRC) of the University of Illinois at Urbana - Champaign. Figure 3.2 shows an integrated system that was proposed for swine manure treatment including the pretreatment of raw manure to produce LPAM, which was subsequently fed to an algal bioreactor. The LPAM production system is composed of a) sedimentation, b) screening, c) bag filtration, and d) microfiltration.

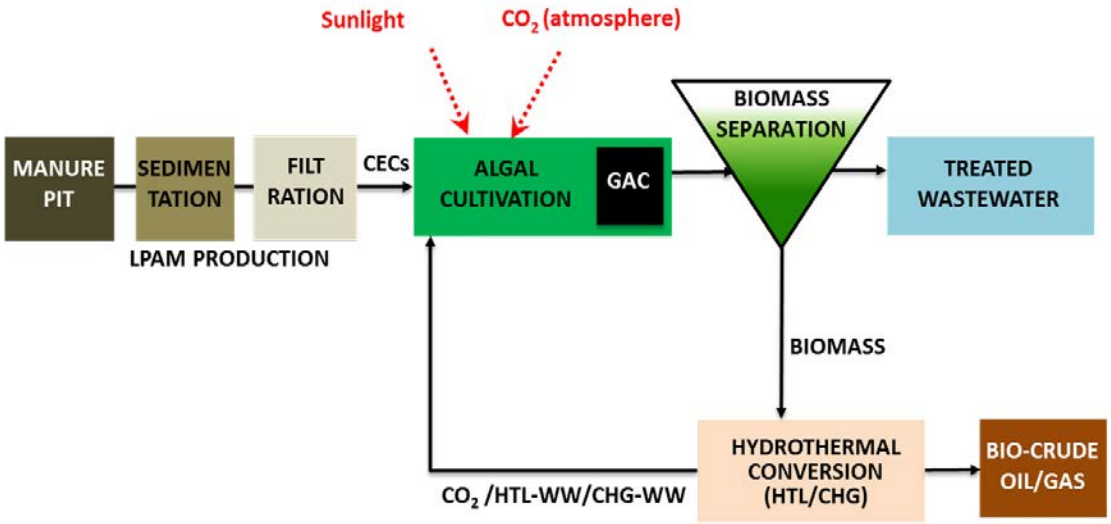


Figure 3.2 Flow diagram of an integrated swine manure management system

3.2.3 DEVELOPMENT OF ANALYTICAL METHODS FOR ESTROGENIC HORMONES

3.2.3.1 SAMPLE PREPARATION

To enhance the sensitivity of analysis by removing the matrix effects, the hormones were extracted and analyzed from both liquid and solid fractions in each step. The final data was

obtained by averaging the results after at least 2 repetitions for both liquid and solid fractions. Fresh swine manure samples were collected from the farrowing barn, and the hormones were extracted from the feces and solid portion of manure slurry followed by centrifugation with methanol. SPE was used to concentrate the target analytes from the extracts by serial methanol extraction, which reduces matrix effects by removing background compounds (Hanselman et al., 2003; Singh et al., 2013; Zheng et al., 2008). The extracts were stored at -20˚C after evaporation with N_2 at 60˚C. Figure 3.3 shows the schematic flow diagram for the sample preparation of liquid and solid fractions.

A) SOLID FRACTION

According to Singh et al., (2013), more than 85% of the spiked hormones could be extracted from solid manure by serial extraction with methanol and sonication, the natural estrogenic hormones in the biomass fraction were extracted using the serial extraction. 10 mL of the effluents from MABB and CAS were centrifuged at 3,500 rpm for 30 minutes to separate the solid and liquid fractions. After decanting the liquid fraction to a glass container, the solid fractions were vortexed with 20 mL of methanol and centrifuged again at 3,500 rpm for 15 minutes to separate the solid and liquid fractions followed by the sonication for 4 minutes. Then, this extraction was repeated 10 times, and all the liquid portion was collected in the glass container (Supelco, Belle-fonte, PA, USA) and stored at - 20°C for hormones analysis.

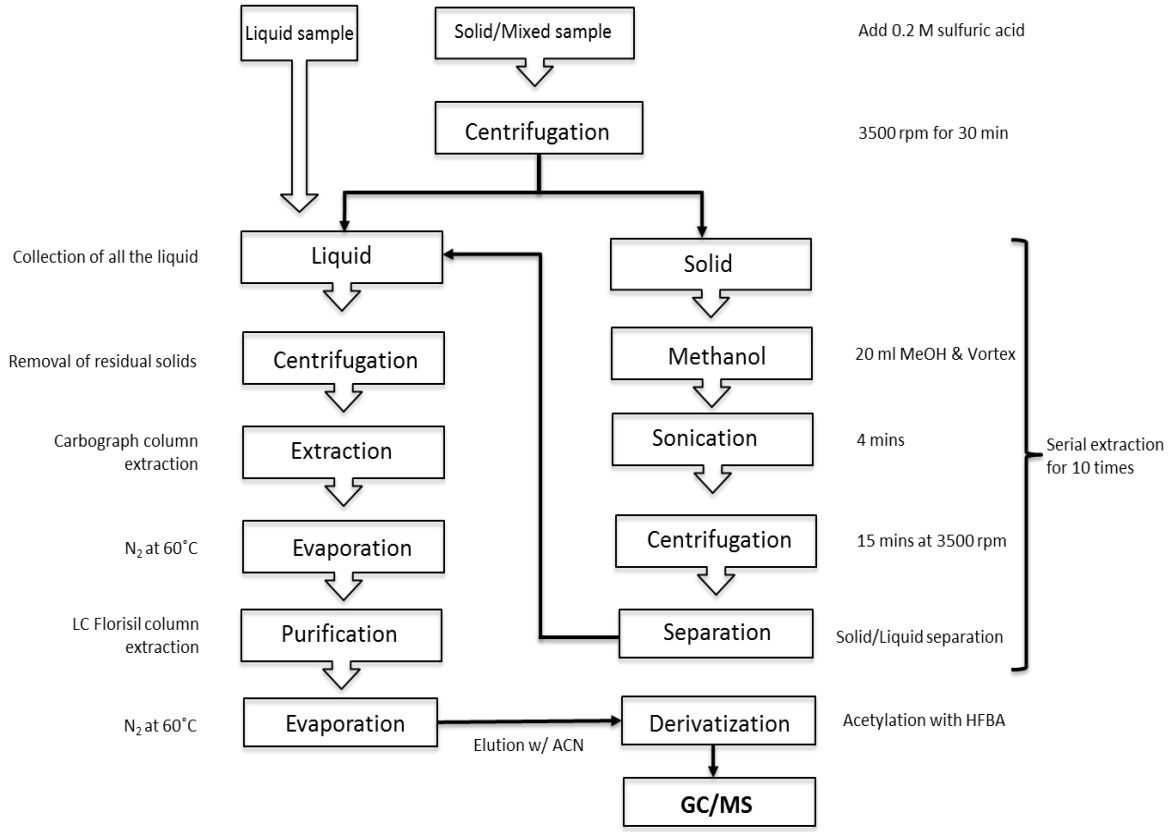


Figure 3.3 Flow diagram of the sample preparation method for estrogenic hormones analysis by GC/MS

B) LIQUID FRACTION

According to the modified method by Zheng et al., (2008), all the hormone samples in the glass vials were extracted and purified by SPE using the Carbograph and Florisil cartridges. As shown in Figure 3.3, the liquid samples were centrifuged at 3,500 rpm for 15 minutes to remove residual suspended solids to minimize Carbograph SPE column plugging. Before passing the liquid portion into the Carbograph column, all the columns were preconditioned by sequentially washing the columns with 10 mL dichloromethane/methanol (80:20), 5 mL of methanol acidified to pH 2 with formic acid, 5 mL of methanol, and 10 mL of DI water. The liquid samples were passed

through the washed Carbograph column using the vacuum (1 drop/sec), and concentrated hormones which was eluted with 10 mL of dichloromethane/methanol (80:20). After evaporating the solvents with N_2 at 60 ˚C, the extracts were re-dissolved in the 5 mL of dichloromethane/hexane (50:50). The concentrates were passed through the Florisil SPE column to clean up the samples for removing matrix effects followed by the preconditioning of cartridge with 5 mL dichloromethane/methanol (80:20) and 5 mL of dichloromethane/hexane (50:50). After additional washing the column with 10 mL of dichloromethane/hexane (50:50), the hormones were eluted with 6 mL of dichloromethane/methanol (80:20) and then the extracts were stored at -20˚C after evaporating the solutions with a gentle stream of nitrogen (N_2) at 60˚C.

After the extraction and purification procedure with SPE, the extracted hormones from each sample were derivatized using acetylation with heptafluorobutyric acid (HFBA) based on the predeveloped and optimized method (Zheng et al., 2008). The extracts were re-dissolved using 500µL of acetonitrile (ACN) followed by adding 50µL of HFBA. The mixture was heated for 1.5 hours at 80°C and cooled to room temperature. After evaporating the solutions with N_2 at 60 ˚C, hexane with hexachlorobenzene (HCB, 100µg/L) was used to re-dissolve the derivatized samples and as internal standard for GC/MS analysis.

3.2.3.2 GC/MS ANALYSIS FOR ESTROGENIC HORMONES

A Hewlett-Packard (HP) 6890 GC in tandem with a Waters AutoSpec Ultima High Resolution mass spectrometer equipped with a Rtx-5MS column (30 m × 0.25 mm i.d. × 0.25-µm film thickness, Restek, Bellefonte, PA) was used for identification and quantitation of steroid hormones. The GC conditions were 1 mL/minute carrier gas flow rate (He), 290°C inlet temperature, and 290°C interface temperature. The initial oven temperature was 80°C for 2

minutes; then the temperature was increased to 230°C at 10°C/minute and held for 3 minutes, and then increased to 280°C at 10°C/minute rate and held for 5 minutes.

Mass spectrometric analysis was carried out using both full scan and selected ion monitoring (SIM) modes. In the full scan mode, the electron impact (EI) mass spectra were generated using an electron energy of 70 eV and ions with *m*/*z* 200–900 were monitored. The full scan mode was used for the identification of analytes by fragmentation patterns and retention time as compared to known standards. For quantitation of hormones in all sample extracts, SIM mode was used. The following quantitation ions were used in the SIM: *m*/*z* = 664.1295 for 17α-estradiol and 17*β*-estradiol, *m*/*z*= 449.1352 for estriol, *m*/*z* = 466.11379 for estrone. For every hormones analysis, five-point calibration curves (10-10000 µg/L) were derived and then analyzed with the internal standard (Hexachlorobenzene). To gauge fluctuation, sample carry over, or possible internal contamination of the instrument, one solvent blank and one standard spike were analyzed between every 15 injections. Spiking 50µg of hormone solution in 1.0 L DI water determined the recovery. 71%-113% represents the recovery rates of the three steroid hormones in DI water. For most hormones, the relative standard deviation (RSD) was less than 5% and the detection limit for E1 and E3 was 20µg/L and 5µg/L for E2 and EE2.

3.2.3.3 CONJUGATED ESTROGENS

Estrogens are excreted as either free estrogens or as sulfate or glucuronide conjugates, with the conjugated forms being biologically inactive. However, estrogen conjugates can be readily hydrolyzed in sewage treatment plants, although the sulfate forms are more recalcitrant than the glucuronide forms. To assess the overall contribution of estrogen conjugates to the total estrogen

loads in the pretreatment process of swine manure, deconjugation using enzymatic hydrolysis was performed followed by analysis of the subsequent increase in free estrogens using GC/MS.

According to Mouatassim-Souali et al., (2003), swine manure samples were analyzed indirectly for estrogen conjugates by subjecting samples to enzymatic hydrolysis using 1 mL of 0.1 M acetic acid buffer (pH=5) containing β-glucuronidase and sulphatase. After 24 hours of incubation at 40°C, the deconjugation reaction was terminated by the addition of acidified water (pH=3). The incubation mixture was then extracted on preconditioned Oasis HLB® SPE cartridges as described previously. The increased level of estrogen concentrations was assessed by comparing the results before and after the enzyme treatment with good reproducibility afterward.

3.2.4 CHARACTERIZATION OF LIQUID PORTION OF ANIMAL MANURE

3.2.4.1 WATER QUALITY ANALYSIS

To investigate the seasonal variation in the water quality parameters of LPAM, swine manure slurry samples were taken at different times. Then, manure slurry samples were filtered using 0.45µm pore size syringe filters (Whatman puradisc-25mm) to remove suspended particles and stored at 4°C to use. Then, soluble chemical oxygen demand (sCOD) was determined by visible light absorbance after dichromate digestion according to standard methods (Clesceri et al. 1999) with a HACH Model DR/2010 spectrophotometer. Total soluble nitrogen was measured using the persulfate digestion method (HACH Method No. 10072). Ammonia nitrogen was measured using salicylate method (HACH Method No. 8155). Total phosphorus was determined by the Molybdovanadate with acid persulfate digestion method for wastewater analyses (HACH Method No. 10127). Total suspended solid was measured following American Public Health Association (APHA) standard methods (APHA. 2005). In addition, the average water quality

parameters of LPAM from shallow/bottom pit were analyzed to check the effects of gravitational sedimentation in the manure pit. All the samples were prepared using the above method and water quality parameters were monitored.

3.2.4.2 ACUTE TOXICITY

Microtox® is a standard toxicity test from Modern Water (New Castle, DE, USA). It is easy and reproducible (Bulich, 1986). This makes it a highly valuable tool for protecting organisms of environmental concern. Acute toxicity can be distinguished from CHO cell cytotoxicity which is a result of adverse health effects from multiple, repeated exposures of a substance, often at lower concentrations, for a longer period (months or years). For bacterial (or CHO cell) toxicity research, it is considered unethical to use human test subjects. Accidental human exposures, however, can contain some useful information (e.g., factory accidents). Otherwise, animal testing or in vitro testing methods and inference of similar substances provides most acute toxicity data.

Microtox testing was performed using a Microtox® model M500 toxicity analyzer and standard procedures recommended by the Modern Water (New Castle, DE, USA). The test is based on the bioluminescence of reconstituted freeze-dried *Photobacterium phosphoreum* (Microtox bacteria) as a measure of biological activity. Light emission by bacteria is decreased by the addition of toxicants in the samples. After five-minute reaction test, relative luminescence light emission was measured by a Microtox® M500 Analyzer (Modern Water, New Castle, DE, USA; formerly SDI). Acute toxicity was expressed by the effective concentration (tEC_{50}) values of wastewater samples, where induced a cell density of 50% as compared to the concurrent negative control. To determine if there was significant difference in acute toxicity among the individual samples, the mean microtoxic bacterial toxicity index (MTI: $tEC_{50}^{-1}\times10^{3}$) and its standard error were calculated

for each tEC_{50} value, and evaluated by student t-test. In this study, to test the acute toxicity of liquid swine wastewater, the acute toxicity assay was used before and after the integrated management procedure for livestock wastewater.

3.2.4.3 OCCURRENCE OF ESTROGENIC HORMONES

To explore the occurrence of natural estrogenic hormones including E1, E2, E3, and EE2 from swine farming systems, fresh samples of raw manure slurry were collected from the gestation, farrowing, and finishing barn in the Swine Research Center (SRC) located at the University of Illinois at Urbana – Champaign (UIUC). The containers and equipment for the sample handling has to be glassware to minimize the loss of hormones. All the samples were transported in ice within 2 hours to the laboratory at UIUC, and immediately extracted. Total and volatile solids of collected manure samples were determined using the standard methods (American Public Health Association, 1998).

3.2.4.4 YES YEAST CELL ASSAY FOR ESTROGENIC ACTIVITY

The YES (Yeast Estrogen Screen) cell assay, yeast-based microplate assay kit (XenoScreen XL YES, Xenometrix, Switzerland) using *Saccharomyces cerevisiae* genetically modified strains with human estrogen receptor (hER), was used to analyze the potential estrogenic activity of estrogenic compounds in the swine manure based on the instruction manual from manufacturer (Xenometrix, XenoScreen XL YES, Instructions for Use Version 1.05). Especially, activating (agonistic) activities of test estrogenic hormones were investigated using the YES assay kit.

The strains, which were included in the XenoScreen XL YES assay, were inoculated and cultured in the YEPD (yeast extract peptone dextrose) growth medium at 31°C with orbital

shaking. After 48 hours of incubation, yeast cells were transformed with either hER (YES) and a b-galactosidase reporter system, and the yeast cell growth was assessed by measuring the optical density at 690 nm (OD_{690}) to calculate the necessary volume of cells to be added to test medium. After serial dilutions of the positive control and samples eluted with dimethylsulfoxide (DMSO), the cultured yeast cells were exposed to test compounds in 96-well plates subsequently adding assay mixture consisted of growth medium containing the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). 17β-estradiol (E2), ranged 10^6 to 10^9 M, and ultrapure water were used as positive and negative control, respectively. The plate was incubated for 48 hours under the same condition of cell culture, and the yeast cell growth was assessed by measuring the optical density in a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, USA) at 690 nm (A_{690}) for growth and at 570 nm (A_{570}) for expression of β-galactosidase, to calculate the growth factor for each well (sample wells compared to solvent control wells). The wells with the highest standard or concentrations should be a deep purple. If needed incubate for another few hours until the color is deep enough. The potential estrogenic activity of the extracts of swine manure samples were evaluated using the calculation of the parameters such as growth factor (G: $A_{690,\ sample} / A_{690,\ solvent}$) and induction ratio ($I_R$: $1/G \times A_{570,\ sample} / A_{570,\ solvent}$) with the absorbance at 690 nm and 570 nm. The calculation of induction or inhibition was corrected according to the growth factor for each well, and therefore the activity of the b-galactosidase was not influenced by inhibited growth of yeast cells. An E2 dose-response curve can be used as a reference by interpolating the response of a suitable concentration to determine the estrogenic potency of samples. A suitable concentration is a concentration response which fits onto the linear E2 dose-response curve. Estradiol equivalents (EEQ) represents the estrogenic potency of the compound or environmental extract as E2. The media effective concentration (EC_{50}) values, in which the concentration of 50%

of the highest estrogenic effect was detected, was used to express the estrogenic potency of a compound relative to E2.

3.2.5 FATE AND TRANSPORT OF CECS IN THE PRETREATMENT PROCESS

3.2.5.1 SEDIMENTATION AND SCREENING

To investigate the effects of sedimentation on the E1, E2, E3, and EE2 concentration, fresh manure slurry samples were analyzed after the gravitational sedimentation in the manure pit and inclined screening test. The manure slurry samples from the surface and sludge layer of the manure storage pit were collected to inspect the effects of sedimentation in the manure pit after 4 days of resting time because gravitational sedimentation of solids and phosphorus could be occurred during the 3 to 7 days of resting time of swine manure slurry in the manure pit before scraping and transporting manure slurry to the lagoon (International Conference on Environmental et al., 2010). Then, the inclined screening technique was used to separate most of the solid portion from the raw swine manure slurry followed by the gravitational sedimentation. Concentrations of E1, E2, E3, and EE2 were analyzed before and after the screening of the settled manure slurry.

3.2.5.2 BAG FILTRATION AND MICROFILTRATION

The effects of microfiltration on the fate of hormones were explored in the process of LPAM production. A chlorinated polyethylene membrane (Kubota Membrane USA Co., cartridge type 203) with nominal 0.4μm pores was used to produce LPAM which is the pure liquid portion of raw swine manure slurry without suspended solids followed by bag filtration with 1.0μm pore size polypropylene filter bag (Filter Specialists, INC., BPOMF X01). The inputs and outputs of

microfiltration system were taken to analyze the estrogenic hormones, and processed the sample preparation based on the methods in Figure 3.3.

3.3 RESULTS AND DISCUSSIONS

3.3.1 OCCURRENCE OF ESTROGENIC HORMONES

3.3.1.1 SOLID MANURE ANALYSIS

To evaluate the serial extraction for analysis the solid fraction, estrogenic hormones were extracted from fresh feces sampled from a farrowing barn using the previously described extraction methods, and the resulting data is shown in Figure 3.4. After increasing the total amount of methanol used in the extraction from 40 to 200 mL and then to 300 mL, the total extracted amount of hormone (µg) per L feces was increased from 1.3µg to 66.6µg and then to 69.3µg, respectively (Figure 3.4). This result demonstrated that the serial extraction method contributed to increase the extracted amount of total estrogenic hormones up to 53 times more (1.3 to 69.3µg per L feces) from 40 to 300 mL of methanol dose, and plateaued for methanol doses higher than 200 mL, which can be used as an optimal dose of methanol for the hormone analysis of solid fraction in the samples.

In addition, natural estrogenic hormones in the solid manure from the gestation, farrowing, and finishing barn were extracted using the serial extraction method as described above followed by GC/MS analysis. The concentration of total extracted estrogenic hormones per solid as follows: gestation (190.1µg/L) > farrowing (66.2µg/L) > finishing (61.7µg/L). Also, the dominant hormones in the gestation, farrowing, and finishing operation solids were E3 (91.9%), E2 (81.3%), and E3 (77.2%), respectively.

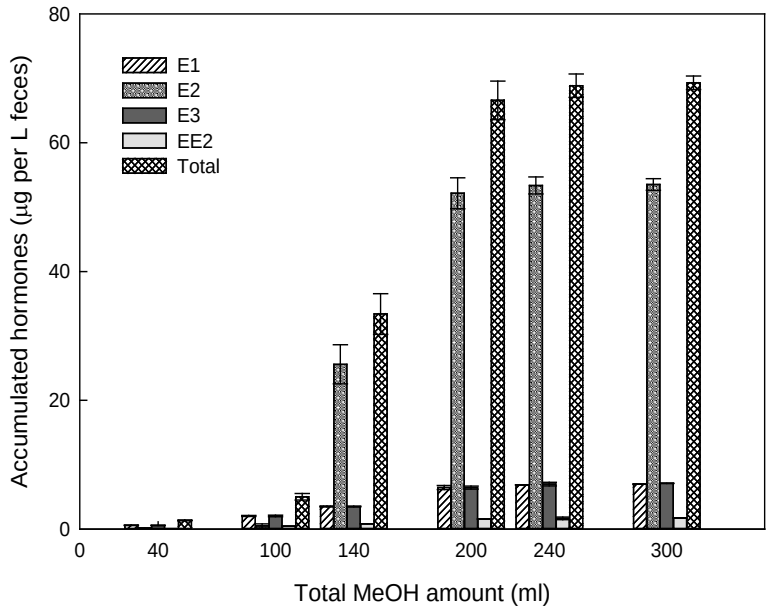


Figure 3.4 Concentrations of estrogenic hormones in a solid manure after serial extraction with methanol. The error bars indicate the standard error of the mean

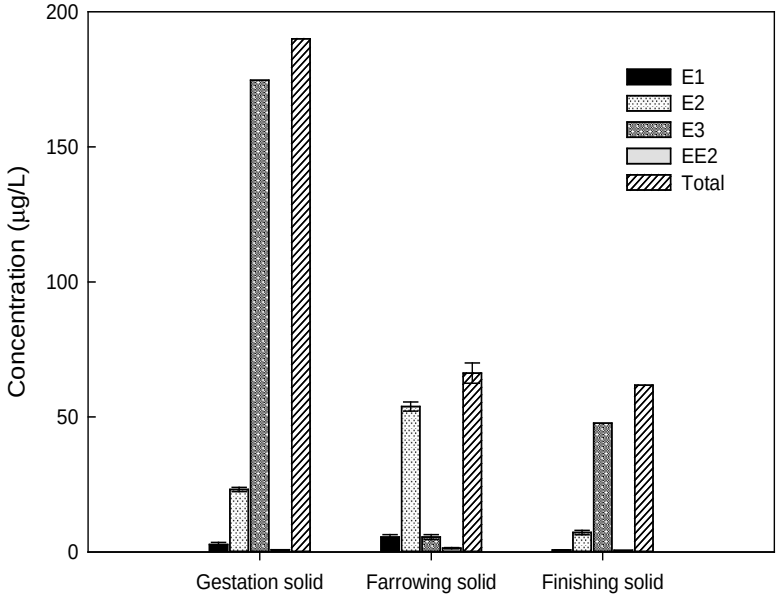


Figure 3.5 Concentrations of estrogenic hormones in a solid manure after serial extraction with methanol. The error bars indicate the standard error of the mean

3.3.1.2 URINE ANALYSIS

Fresh urine samples from the gestation, farrowing, and finishing barns were analyzed for the estrogenic hormones using the previously described methods. The concentrations of E1, E2, E3, and EE2 in the urine samples are in Table 3.2, and total amount of hormones in urines as follows: farrowing (22.9μg/L) > gestation (5.8μg/L) > finishing (1.8μg/L). The dominant hormones in the urine of gestation, farrowing, and finishing were E1 (43.4%), E3 (45.9%), and E2 (84.6%), respectively.

Figure 3.6 demonstrates that most of the free estrogens are fecal estrogens and excreted in free forms from livestock animals, which is consistent with the previous studies (Combalbert et al., 2012; Hanselman et al., 2003; Zhang et al., 2014). The gestation barn was the biggest producer of natural estrogens in the swine farm, which was 3 to 4.2 times more than farrowing and finishing barn. However, previous data comparing fecal and urinary estrogens from different types of swine operations (gestation, farrowing, finishing) were quite limited. This study would be good reference data for calculating the overall estrogen production rate of a swine farming system.

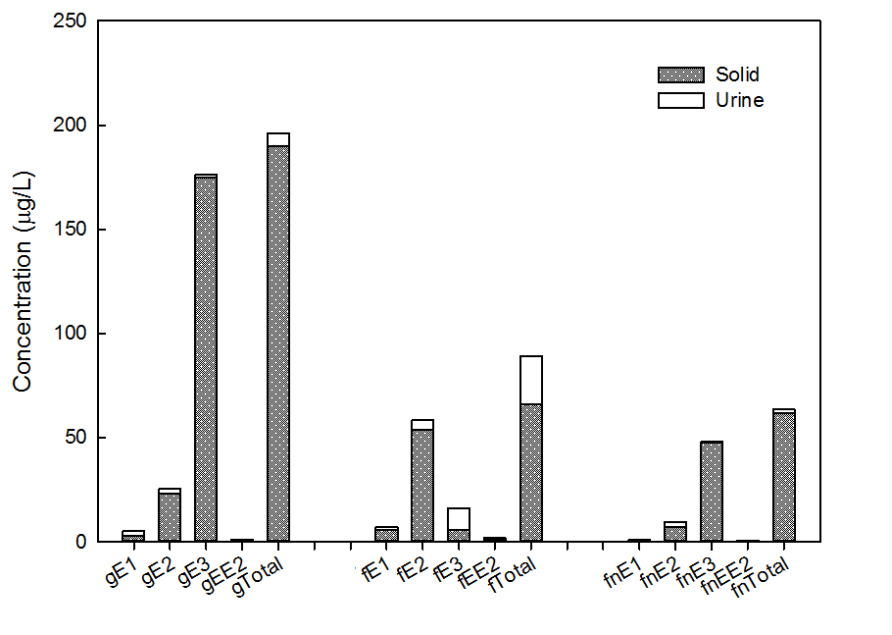


Figure 3.6 Distribution of estrogenic hormones in the urine and solid from gestation, farrowing, and finishing barn

Notes: g + estrogenic hormones: hormones concentration in the sample from gestation barn
f + estrogenic hormones: hormones concentration in the sample from farrowing barn
fn + estrogenic hormones: hormones concentration in the sample from finishing barn

3.3.1.3 MANURE SLURRY ANALYSIS

As shown in the Figure 3.7, natural E1 and E2 concentrations in the manure slurry samples from the farrowing, gestation, and finishing barns were analyzed and compared to previous studies to evaluate extraction process for manure slurry and the occurrence data of hormones. The concentrations of E1 and E2 in all the manure slurry ranged from 756 to 3,135µg/L and 4,135 to 14,762µg/L, respectively, which is much higher than results from the previous literature (Hanselman et al., 2003; Raman et al., 2004; Sim et al., 2011; Singh et al., 2013). This suggests that the serial extraction technique used for estrogen analysis of manure samples in this study was

more effective for extracting estrogenic hormones from the solid fraction, and it could enhance the analytical results in future hormonal analyses of solid and liquid manure mixtures.

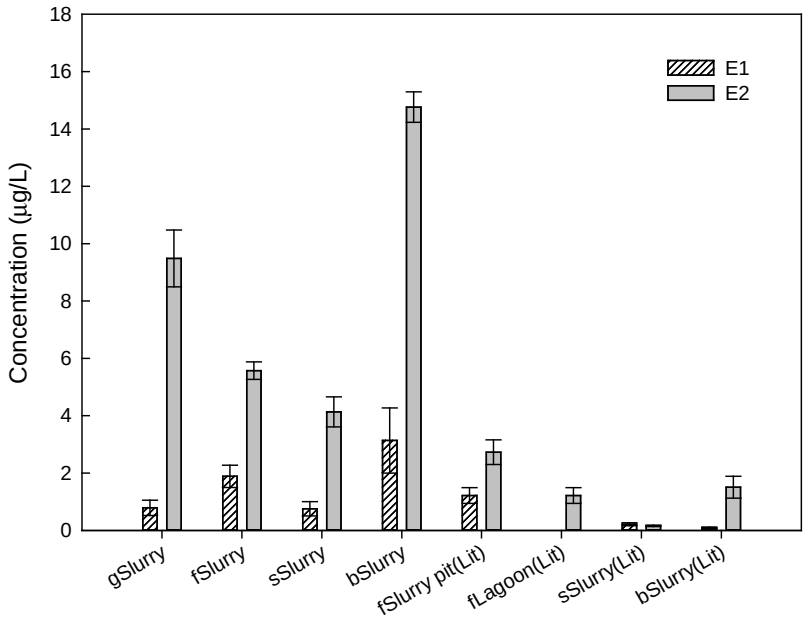


Figure 3.7 Estrogenic hormones in manure slurry from gestation, farrowing, finishing barn, and literatures. The error bars indicate the standard error of the mean

Note: gSlurry: Slurry produced from the gestation barn

fSlurry: Slurry produced from the farrowing barn

sSlurry: Slurry produced from the surface of the finishing manure pit

bSlurry: Slurry produced from the bottom of the finishing manure pit

fLagoon: Livestock wastewater from finishing lagoon

Based on these results, daily production of estrogenic hormones from different barns were calculated to evaluate the environmental benefits of the proposed integrated manure management systems. SRC provided the number of pigs in each barn, and the average daily production of mixed manure slurry composed of feces, urine, and cleaning waters were found in the previous studies (Curran. 2015). As shown in Table 3.2, the daily production of total estrogens in the farrowing

barn was 106,664 ± 1,549 µg/day, which is 40.8% and 2.9% higher than gestation and finishing barns, respectively. Total hormones per pig were as follows: farrowing (1,333 ± 19µg/day-hd) > gestation (789 ± 15µg/day-hd) > finishing (518 ± 5µg/day-hd).

In addition, the average daily production of E1, E2, E3, and EE2 in the total manure slurry from SRC as follows: E3 (162,447 ± 2,266µg/day) > E2 (59,600 ± 621µg/day) > E1 (36,211 ± 359µg/day) > EE2 (15,044 ± 195µg/day). In conclusion, 759.2 ± 10.5µg of estrogenic hormones was produced each day per pig from SRC. With these starting concentrations defined, the feasibility and efficiency of using the proposed integrated manure management system for the removal of estrogens has to be investigated to see if it meets the targeted hormones concentration, which is ≤ 10ng/L.

Table 3.2 Daily production rate of estrogenic hormones from gestation, farrowing, and finishing barn

	Manure type	Average pigs (hd)	Manure production rate (L/hd/d)	E1	E2	E3	EE2	**Total**
Gestation barn	Mixed Slurry (μg/day)	80	52.9	8,339 ± 98	10,987 ± 37	42,435 ± 1,185	1,348 ± 15	**63,110 ± 1,166**
	Per pig (μg/day-hd)			104.2 ± 1.2	137.4 ± 0.5	530.4 ± 14.8	16.9 ± 0.2	**788.9 ± 14.6**
Farrowing barn	Mixed Slurry (μg/day)	80	41.6	12,029 ± 145	23,606 ± 356	58,661 ± 522	12,366 ± 169	**106,664 ± 1,549**
	Per pig (μg/day-hd)			150.4 ± 1.8	295.1 ± 4.5	733.3 ± 6.5	154.6 ± 2.1	**1,333.3 ± 19.4**
Finishing barn	Mixed Slurry (μg/day)	200	20.8	15,842 ± 116	25,005 ± 227	61,350 ± 559	1,330 ± 11	**103,528 ± 1,072**
	Per pig (μg/day-hd)			79.2 ± 0.6	125 ± 1.1	306.8 ± 2.8	6.7 ± 0.1	**517.6 ± 5.4**
Total	Mixed Slurry (μg/day)	360	115.4	**36,211 ± 359**	**59,600 ± 621**	**162,447 ± 2,266**	**15,044 ± 195**	**273,302 ± 3,787**
	Per pig (μg/day-hd)			**100.6 ± 1.0**	**165.6 ± 1.7**	**451.2 ± 6.3**	**41.8 ± 0.5**	**759.2 ± 10.5**

3.3.2 EFFECTS OF SEDIMENTATION AND FILTRATION ON THE FATE OF HORMONES

3.3.2.1 GRAVITATIONAL SEDIMENTATION AND INCLINED SCREENING

Figure 3.8 (A) and (B) showed the effects of sedimentation on the nutrients movement in the storage pit, and the average water quality parameters of liquid manure from the surface/bottom pit were as follows: sCOD - 4,406/36,380mg/L, TDN - 1,183/2,433mg/L, TDP - 194/233 mg/L, and NH_3-N - 235/537mg/L. Overall concentrations of water quality parameters were doubled after sedimentation process, but sCOD increased up to 8.3 times. These results were coincident with the previous studies about the nutrient sedimentation in the manure storage pit (International Conference on Environmental et al., 2010). In Figure 3.8 (B), the total solids and volatile solids of the bottom manure slurry (bSlurry) increased up to 55.4% and 56.9% in the storage pit, which demonstrates that gravitational sedimentation contributed to separate the solids from the manure slurry efficiently without further energy input. According to Figure 3.8 (C) and Table 3.3, after 4 days of gravitational sedimentation of swine manure slurry, the concentrations of E1, E2, E3, and EE2 in the manure slurry from the bottom pit (bSlurry) were 1.5 to 3.8 times higher than the concentrations of the surface slurry (sSlurry). This demonstrates that estrogenic hormones could be accumulated on the bottom of the pit by the settling of the solids in the storage pit due to the hydrophobicity of the free estrogenic hormones.

In conclusion, the gravitational sedimentation in the storage pit was effective as a pre-treatment of manure slurry to extract solids, nutrients, and estrogenic hormones without energy input. However, the drawbacks of gravitational sedimentation are adequate space to keep the manure during the settling and required retention time to settle out the slurry. To overcome these limitations and accelerate the sedimentation, an inclined screening process was tested with the bottom slurry followed by the sedimentation process.

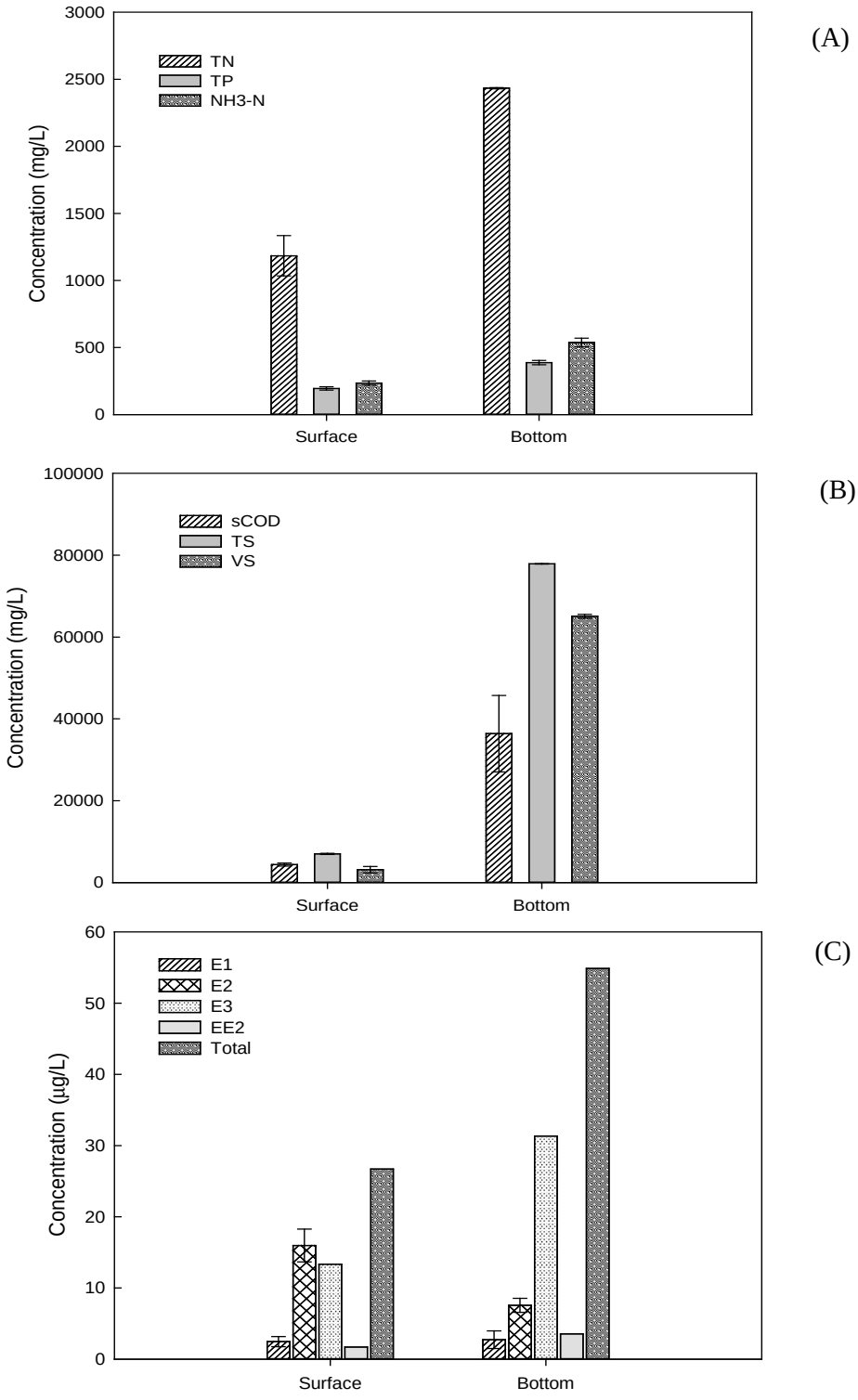


Figure 3.8 Effects of sedimentation on (A) nutrients (TN/TP/NH_3-N), (B) sCOD/TS/VS, and (C) estrogenic hormones. The error bars indicate the standard error of the mean

After the inclined screening of the settled slurry, the solids content and estrogenic hormones were analyzed to investigate the effects of inclined screening on the distribution of solids and hormones. Table 3.3 and Figure 3.8 (C) noted that the percent removal of total and volatile solids was 19% and 21%, and the removal rate of total and volatile solids in the inclined screening was about 34 and 30mg/L-d, respectively. Furthermore, the percent removals of estrogenic hormones ranged from 0.4 to 24% in Figure 3.9 (B). Based on these results, inclined screening was highly effective to remove the solids content and estrogenic hormones in the swine manure slurry along with the short processing time.

Table 3.3 is a summary table that shows the removal of solids content, dissolved organics and nutrients, and estrogenic hormones in the manure slurry before and after each process.

Table 3.3 Effects of sedimentation and screening on the concentrations of E1, E2, E3, and EE2 in the manure slurry

	SEDIMENTATION		FILTRATION	
	Gravitational sedimentation	Inclined screening	Bag filtration	Micro filtration
Solid content (g/L)				
Total Solid	77.9 ± 0.4	63.1 ± 0.4	13.8 ± 0.1	3.5 ± 0.4
Volatile Solid	65.1 ± 0.5	51.2 ± 0.3	8.1 ± 0.2	1.6 ± 0.2
Solid removal percentage (%)				
Total Solid	79.8 ± 0.4	19.0 ± 0.5	82.3 ± 0.1	95.5 ± 0.1
Volatile Solid	88.8 ± 2.8	21.4 ± 0.5	89.6 ± 0.02	98.0 ± 0.0
Solid removal rate (g/L-d)				
Total Solid	2.2 ± 0.08	33.8 ± 0.08	42.1 ± 0.07	0.03
Volatile Solid	1.6 ± 0.05	29.9 ± 0.08	35.8 ± 0.06	0.02
Estrogenic hormones (µg/L)				
Estrone (E1)	3.1 ± 1.1	16.8 ± 0.5	0.4 ± 0.1	2.7 ± 1.2
17β-estradiol (E2)	14.8 ± 0.5	6.9 ± 0.1	1.2 ± 0.1	7.6 ± 1.0
Estriol (E3)	18.7	0.6	0.3	31.3
17α-estradiol (EE2)	0.4	0.1	0.1	3.5
Total	37.0	24.4	2.0	45.2
Estrogenic hormones removal (%)				
Estrone (E1)	48.94 ± 4.4	-441.9 ±55.2	97.7 ± 0.02	-610.5 ± 78.9
17β-estradiol (E2)	46.5 ± 1.7	53.4 ± 0.4	82.6 ± 0.4	-533.3 ± 25
Estriol (E3)	14.3	96.8	48.3	-10,004.6
17α-estradiol (EE2)	25.8	67.5	23.1	-3,440
Total	25.4	34.0	91.9	-2,169.9
Hormones removal rate (µg/L-d)				
Estrone (E1)	0.1 ± 0.01	-43.6 ± 2.7	46.7 ± 1.4	-0.03 ± 0.2
17β-estradiol (E2)	0.3 ± 0.07	36.2 ± 1.7	17.2 ± 0.1	-0.09 ± 0.1
Estriol (E3)	1.6	72.9	1.1	-0.44
17α-estradiol (EE2)	0.1	1.2	0.2	-0.05
Total	2.1	66.7	65.2	-0.61

3.3.2.2 BAG FILTRATION AND MICROFILTRATION

To investigate the effects of bag filtration and microfiltration of manure slurry on the fate of estrogenic hormones, the concentration of E1, E2, E3, and EE2 in the slurry before and after each process were analyzed and compared to evaluate the performance of each process. After bag filtration of screened manure slurry (sbSlurry), 19% and 21.4% of total and volatile solids were removed (Table 3.3). And the percent removal of E1, E2, E3, and EE2 in the bag filtered slurry (bbSlurry) were 97.7, 82.6, 48.3, 75, and 91.9%, respectively (Table 3.3 and Figure 3.9 B). Bag filtration method was faster than the other methods to remove the solids content and effective to remove estrogenic hormones as much as screening.

Finally, the microfiltration was applied to the bag filtered manure slurry, and it contributed to remove total solids and volatile solids of up to 95.5 and 98%. However, the concentrations of all the estrogenic hormones in the microfiltered slurry (LPAM) sharply increased up to 104 times higher than that of bag filtered slurry, which correlates to a percent removal of -1,004.6% in E3. Because the concentrations of estrogens were increased after removing remove most of the suspended solids (mbSlurry: 0–30mg/L, TSS) from the bbSlurry, this increase is note related to the removal of suspended solids. This unexpected increase of estrogens will be explained in next paragraph based on the previous references.

3.3.2.3 RELATIONSHIP BETWEEN SOLIDS DISTRIBUTION AND HORMONAL TRANSPORT

Figure 3.9 showed the transport and distribution of manure solids in the pretreatment process, which includes the sedimentation, screening, bag- and micro-filtration to separate the solid fraction from manure slurry. Figure 3.9 also described the concentration of E1, E2, E3, and EE2 in the manure slurry before and after each pretreatment process. The results demonstrated that

overall amount of estrogens was decreased as the total solids were removed in the pretreatment process. Because the free estrogens are hydrophobic compounds which can be easily absorbed to the solid fraction in the manure slurry, the amount of free estrogens in the liquid portion could be decreased. However, E3 concentration was sharply increased after the microfiltration, and this can be explained by the deconjugation of E3–glucuronide in the manure slurry.

According to Zhang et al., (2014), urinary estrogens are mostly conjugated forms, which are 98 - 99% of total urinary estrogens. And Singh et al., (2013) noted that and the concentration of conjugated E3 in the liquid manure was 155 ± 37µg/L, which was 69 to 112 times higher than total free estrogens (1.7 ± 0.1µg/L) in the bag filtered manure slurry, because the E3-glucuronide has higher solubility in water (812mg/L) than free E3 (3.2 – 13.3mg/L). Louis Cohen et al., (1935) described that E3–glucuronides were completely hydrolyzed by prolonged exposure to bacteria on pregnancy urine, and Belfroid et al., (1999) used fecal *E. coli* to induce the hydrolysis of conjugated estrogens. Furthermore, Venning, (1950) found that incubation of urines with bacteria caused significant hydrolysis of glucuronide estrogens, and several previous studies indicate that glucuronide conjugated estrogens were rapidly deconjugated (Ternes et al., 1999; Vos. 1996). However, sulfate conjugated estrogens may be more resistant to deconjugation, and E3-sulfate was more stable with a lag phase of 70 hours for hydrolysis (D'Ascenzo et al., 2003; Vos. 1996; Zhang & Henion. 1999). Therefore, the E3–glucuronide can be deconjugated to free E3 by the prolonged bacterial hydrolysis in the swine manure slurry because the microfiltration of manure slurry was

relatively slow. In conclusion, deconjugation of E3–glucuronide contributed to increase E3 concentration in the liquid portion of mbSlurry even though most of the solids were removed.

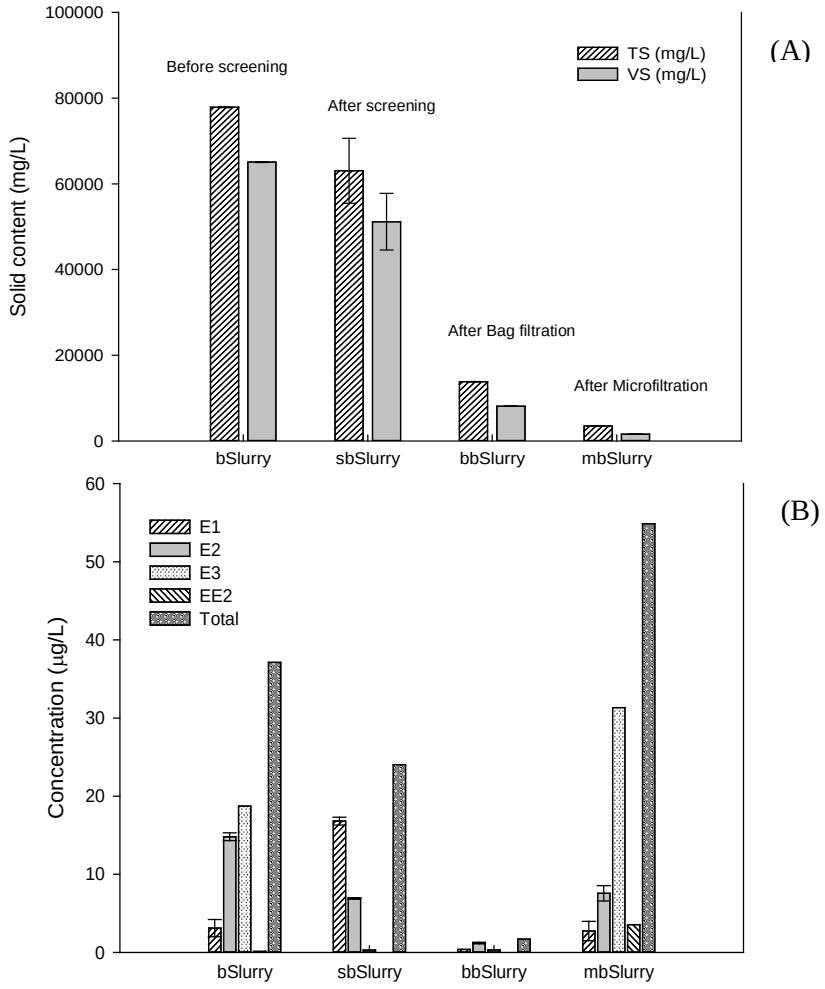


Figure 3.9 (A) TS and VS of manure liquid in the sedimentation and filtration process (B) Effects of sedimentation and filtration on the estrogenic hormones in manure liquid. The error bars indicate the standard error of the mean

Note: bSlurry: Slurry after the gravitational sedimentation in the finishing manure pit

sbSlurry: bSlurry after screening

bbSlurry: bSlurry after bag filtration

mbSlurry: bSlurry after microfiltration

3.3.3 CHARACTERIZATION OF LIQUID PORTION OF ANIMAL MANURE

3.3.3.1 ESTROGENIC HORMONES ANALYSIS

The concentration of estrogenic hormones in the LPAM from gestation, farrowing, and finishing barn were analyzed to investigate the residual estrogens after pre-treatment of different types of manure slurry. In Table 3.4, the concentration dominant hormones (E1, E2, and E3) in each LPAM were ranged from 0.3 to 25.4µg/L, 1.3 to 30.9µg/L, and 0.2 to 175µg/L which are within the range of previous reports; 0.13 to 311.8µg/L (Hanselman et al., 2003; Raman et al., 2004; Sim et al., 2011; Singh et al., 2013). Even though, after removing the suspended solids from the manure slurry samples, the concentration of most estrogens in the LPAM samples had increased compared to the original slurry samples. Several previous studies described that the biological transformations between E1, E2, E3, and EE2 could increase the concentrations of the other compounds during the livestock or municipal wastewater treatment processes (Casey et al., 2005; Ghasemi et al., 2011; Isabelle et al., 2011; Shi et al., 2013; Zheng et al., 2012). However, biological transformation is not sufficient to explain this results as the total estrogen concentration in the LPAM were increased up to 75.6% after pretreatment in this study. Therefore, the biological deconjugation of glucuronide or sulfate estrogens can be suggested to clarify the overall amount of elevated estrogens. Especially, glucuronide estrogens may have contributed towards the increase of estrogens in the LPAM because deconjugation of glucuronide estrogens are relatively faster and susceptible than sulfate estrogens (D'Ascenzo et al., 2003; Ternes et al., 1999; Vos. 1996; Zhang & Henion. 1999).

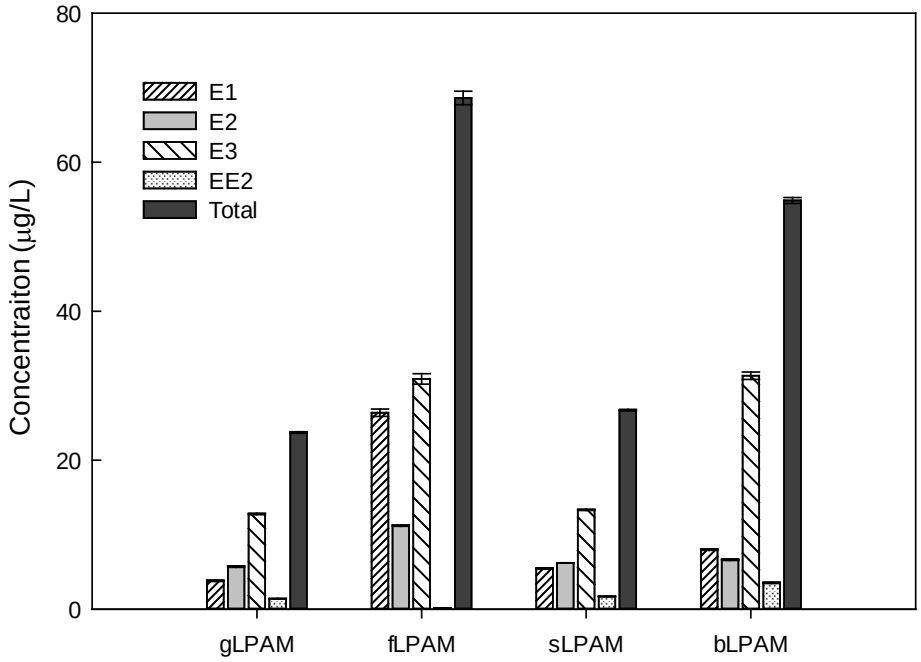


Figure 3.10 Estrogenic hormones in LPAM samples from gestation, farrowing, surface, and bottom LPAM in finishing barn. The error bars indicate the standard error of the mean

Figure 3.10 showed the characterization of estrogenic hormones in LPAM samples from gestation, farrowing, and surface/bottom of the finishing barn. As previous results, LPAM of farrowing barn contain the biggest amount of hormones. E3 was the dominant hormone in all the LPAM samples which supports the previous results of sharp increase of E3 after LPAM production process in Figure 3.9.

Table 3.4 Concentrations of estrogenic hormones in manure slurry and LPAM from gestation, farrowing, and finishing barn

Parameter	Unit	Gestation barn		Farrowing barn		Finishing barn			
		gSlurry	gLPAM	fSlurry	fLPAM	sSlurry	sLPAM	bSlurry	bLPAM
E1		2.0 ± 0.02	3.7± 0.1	3.6 ± 0.04	25.4 ± 0.5	1.8± 0.03	5.6 ± 0.1	7.8 ± 0.1	28.3 ± 0.1
E2		2.6 ± 0.01	5.8 ± 0.1	7.1 ± 0.1	11.7 ± 0.1	3.8 ± 0.1	6.2 ± 0.01	10.4 ± 0.1	12.4 ± 0.1
E3	**µg/L**	10.0 ± 0.3	12.9 ± 0.1	17.6 ± 0.2	29.6 ± 0.7	12.8 ± 0.2	13.1 ± 0.1	18.6 ± 0.1	30.3 ± 0.5
EE2		0.3 ± 0.01	1.4 ± 0.02	3.7 ± 0.1	0.2 ± 0.02	0.4± 0.01	1.8 ± 0.04	0.1 ± 0.02	3.8 ± 0.1
Total		14.9 ± 0.3	23.8 ± 0.1	32.0 ± 0.5	66.8 ± 0.9	18.8 ± 0.3	26.7 ± 0.1	36.9 ± 0.1	54.8 ± 0.4

a. gLPAM/gSlurry: LPAM/Slurry produced from the gestation barn

b. fLPAM/fSlurry: LPAM/Slurry produced from the farrowing barn

c. sLPAM/sSlurry: LPAM/Slurry produced from the surface of the finishing manure pit

d. bLPAM/bSlurry: LPAM/Slurry produced from the bottom of the finishing manure pit

3.3.3.2 YES YEAST CELL ASSAY FOR ESTROGENIC ACTIVITY

After 48 hours of incubation, an eight-point standard E2 curve (0.003 to 2.72µg/L) on a microplate (Left two lanes in Figure 3.11 (A)) should be a deep purple from the bottom wells and going to be yellow with dilutions. This standard curve was used to calculate the E2 equivalents in the YES test. EC_{50} value of E2 standard curve was shown to reside in the microgram per liter range (EC_{50}: 0.11µg/L). Antagonistic estrogenic activities were detected from gLPAM, fLPAM, sLPAM, and bLPAM (Figure 3.11 (B)), and EC_{50} of four LPAM samples ranged from 0.02 to 3.2µg/L. In Figure 3.11 (C), EEQ of four different LPAM samples ranged from 28.2 to 191.5µg/L, which showed the equivalency to E2 stock solution. The LPAM from the bottom of manure pit was the most potent sample compared to the other LPAM and had the highest EEQ as well (Figure 3.11 (B) and (C)).

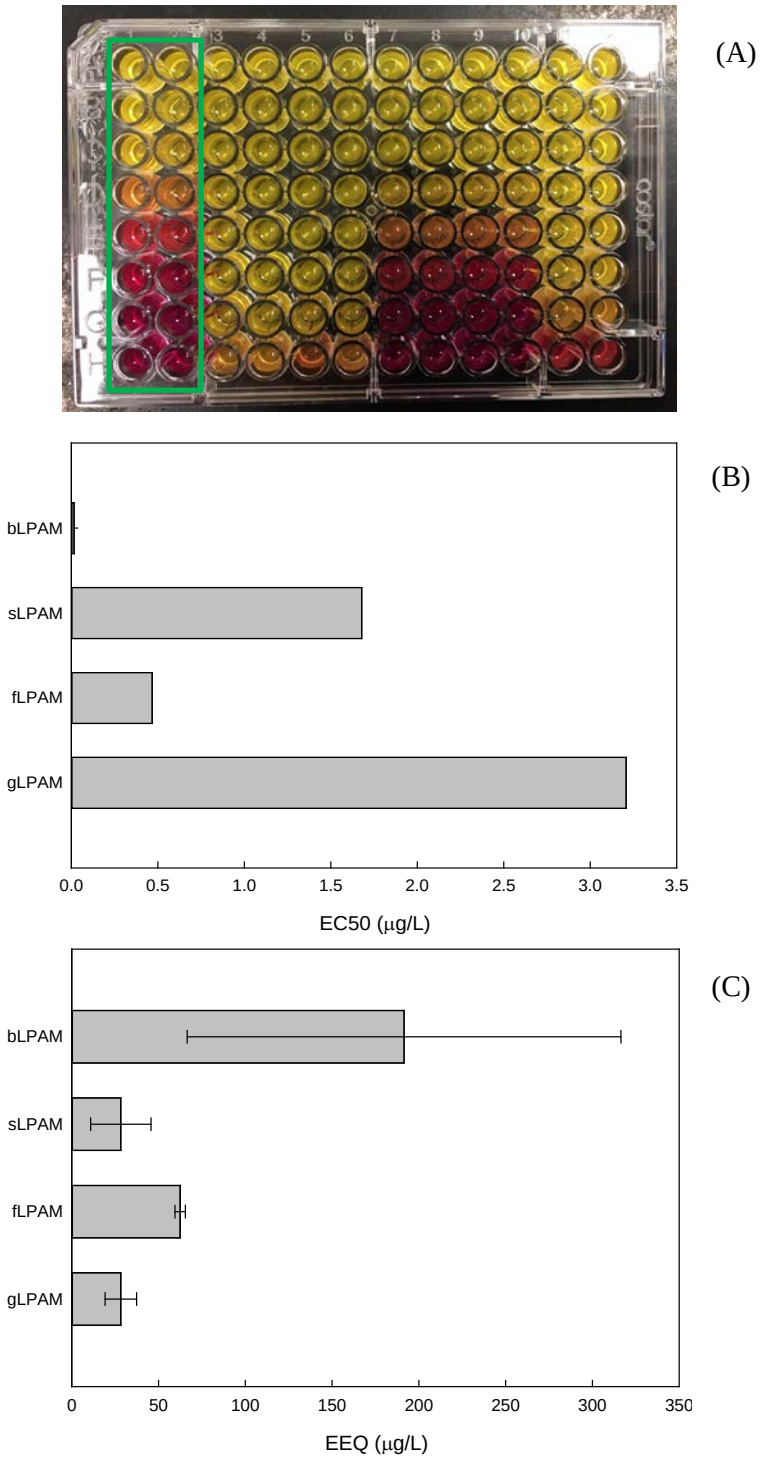


Figure 3.11(A) Plate showing the response of the yeast screen of samples (B) EC50 for agonistic activities and (C) EEQ of hormone compounds in the different types of LPAM (bLPAM, sLPAM, fLPAM, and gLPAM). The error bars indicate the standard error of the mean

3.3.3.3 WATER QUALITY PARAMETERS

In Figure 3.12, the concentrations of sCOD, TN, and TP in the LPAM (mbSlurry) generally increased 2 to 3 time more at between Nov 2013 and July 2014 along with the elevation of air temperature from -1.6°C to 23.9°C, which implied the seasonal variation of the water quality parameters. The averages of standard water quality parameters such as sCOD, TDN, TDP, TOC, NH_3-N, TS, and VS in the LPAM were 3934, 1540, 233, 1703, 235, 3485, and 1596mg/L, respectively. International Conference on Environmental et al., (2010) demonstrated that phosphorus was more easily sedimented than other nutrients, and Stafford and Collison, (1987) noted that maximum and minimum pit temperature ranged from 0.6°C to 33.3°C under seasonal variation, respectively. Thus, as reported by Rieck-Hinz, (2003), the different storage temperature in summer versus winter could affect the nutrient level in the manure slurry. Therefore, these tests proved that there were seasonal variations in water quality parameters because of temperature fluctuation.

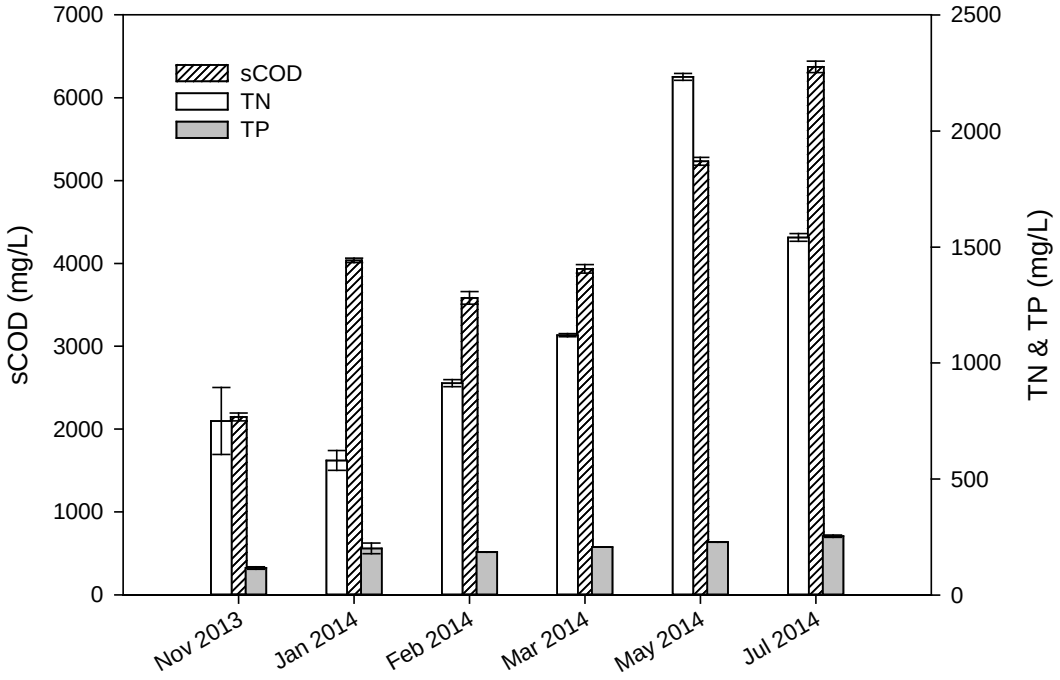


Figure 3.12 Seasonal variation in the standard water quality parameters of LPAM. The error bars indicate the standard error of the mean

3.3.3.4 ACUTE TOXICITY

To characterize the acute toxicity of LPAM from the different types of barn (gestation, farrowing, and finishing barn), the acute toxicity of several LPAM samples were investigated using a Microtox® assay kit, which is commercially available. Figure 3.13 showed that the MTI value of fLPAM was 6,443, which was the highest toxicity compared to the others. Thus, bLPAM was more toxic compared to sLPAM because of sedimentation for organics, nutrients, and heavy metals that can affect the acute toxicity. This result could be supported by the Figure 3.8, which is about the increased concentrations of dissolved organics, nutrients, and suspended solids due to the sedimentation effects in the manure pit.

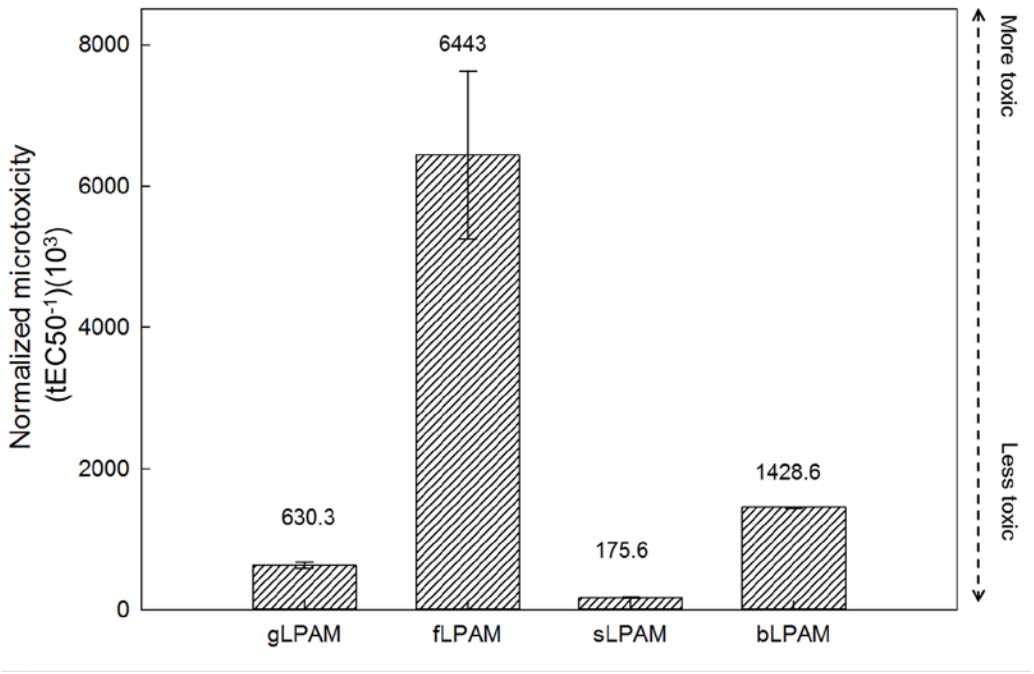


Figure 3.13 Acute toxicity of different types of LPAM via Microtox® assay. The error bars indicate the standard error of the mean

3.4 CONCLUSIONS

The serial extraction with methanol increased the extracted amount of total estrogenic hormones from feces by 53 times and, it plateaued at 200 mL methanol, which demonstrated that 200 mL of methanol, which could be used as an optimal dose for the hormone analysis of solid fraction in the samples. The extracted hormones from gestation solids contained 67.5% higher than the other solids. In addition, the dominant hormones in the solid manure from the gestation, farrowing, and finishing barn were E3 (91.9%), E2 (81.3%), and E3 (77.2%), respectively. The total amount of estrogens in the farrowing urine was up to 22.9 µg/L, which was 74.6 to 92.1% higher than the gestation and finishing urines, respectively. The results of estrogen analysis in the solid and liquid manure demonstrated that most of the free estrogens are fecal estrogens, which is

consistent with the previous studies (Combalbert et al., 2012; Hanselman et al., 2003; Zhang et al., 2014).

The gestation barn was the biggest producer of natural estrogens in the swine farm, with results that were 3 to 4.2 times more than the farrowing and finishing barns. Based on the occurrence data of estrogens, the calculated average daily production of estrogens per pig was 1,333.3 ± 19.4 µg/day-hd in the farrowing barn, which produce 40.8 and 2.9% more than the gestation and finishing barns. The average daily production of E1, E2, E3, and EE2 per pig in the total manure slurry were as follows: E3 (451.2) > E2 (165.6) > E1 (100.6) > EE2 (41.8µg/day-hd). Overall, an average of 759 ± 10µg of estrogenic hormones were produced per pig each day, and this data can be useful to evaluate the feasibility and efficiency of the proposed integrated manure management systems for hormones removal.

As a first step in the pretreatment of manure slurry, gravitational sedimentation in the manure pit was effective for removing solids (65 - 78%), nutrients (17 - 88%), and estrogenic hormones (14 - 49%) without energy input. As an alternative to sedimentation, an inclined screening process was tested with the bottom slurry in the finishing barn. Overall, the percent removal and removal rate of solids were 19 - 21% and 30 - 34g/L-d, and the percent removal of estrogenic hormones ranged from 0.4 to 24%, which was not as effective as sedimentation, but much faster. The screened manure slurry was filtered through a filter bag, and the removal of solids and estrogens ranged from 89 to 93% and 48 to 98%, respectively, which were higher than with the other methods. Thus, bag filtration had the highest removal rate of solids (36 - 42g/L-d) and estrogens (0.2 - 47µg/L-d), which means bag filtration was the fastest and most effective method for the removal of solids and estrogens among the pretreatment processes. As a last step in the pretreatment, the percent removal of solids reached 96 - 98%. However, microfiltration was the

slowest method for the solids removal, and the concentrations of most estrogens had increased up to 104 times higher than bag-filtered slurry. This unexpected increase of estrogens could be explained by a biological deconjugation of the glucuronide estrogens as observed in previous studies, which indicated that glucuronide-conjugated estrogens were rapidly deconjugated (Ternes et al., 1999; Vos. 1996). However, sulfate-conjugated estrogens might be more resistant to deconjugation, and sulfate estrogens were more stable (D'Ascenzo et al., 2003; Vos. 1996; Zhang & Henion. 1999). Therefore, the glucuronide estrogens could be deconjugated to free estrogens by prolonged bacterial hydrolysis due to the relatively slow microfiltration process.

Finally, all the results about solids, estrogenic hormones, and water quality tests supported the feasibility and advantages of the proposed manure pretreatment system over the conventional wastewater treatment process. Also, the data about the removal rates of solids and estrogens could be used to design an effective manure pretreatment system by characterizing the fate and transport of the natural estrogens in the system.

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CHAPTER 4. FATE AND TRANSPORT OF CHEMICALS OF EMERGING CONCERN DURING A MIXED ALGAL-BACTERIAL BIOREACTOR

4.1 INTRODUCTION

Natural and synthetic estrogenic hormones at trace levels in the environment have been reported in numerous studies and are of growing concern due to potential adverse effects on the reproductive biology of vertebrates at very low concentrations (10 - 100ng/L) (Routledge et al., 1998; Schuh et al., 2011). Naturally occurring estrogens in animal waste can cause negative environmental impacts through the disruption of the endocrine systems in wildlife, domesticated animals, and humans (Khanal et al., 2006). Excretion of steroidal estrogens from humans and farm animals is the major source of estrogenic compounds in the environment and can potentially contaminate surface and ground water (Finlay-Moore et al., 2000; Hanselman et al., 2004; Raman et al., 2004; Shore et al., 1993). The European Union and the United States reported an estimated annual excreted estrogen volume from livestock animals as 39 tons and 41 tons, respectively (Lange et al., 2002). High concentrations of estrogenic hormones and their partial breakdown products (BP) are often reported in wastewaters containing manure (Hanselman et al., 2003; Hutchins et al., 2007). Moreover, concentrations of estrogens in wastewater originating from agricultural activities was found to be three to four times higher than municipal wastewaters (Shore et al., 1993). Excretion of estrogenic hormones from agricultural activities is a significant concern for contamination of water resources because they generally receive lower levels of treatment before environmental discharge and can cause adverse ecological effects at very low concentrations (Irwin et al., 2001; Jobling et al., 1998). For example, manure contains bioactive

chemicals that that can contribute to high rates of feminization for aquatic species, thus reducing reproductive abilities, and these chemicals can increase physical deformities by disrupting normal endocrine system function (Irwin et al., 2001; Panter et al., 1998). Therefore, it is particularly important to better understand the fate, transport, and transformation of these bioactive compounds in livestock systems and to develop management practices that cost-effectively mitigate the associated risks.

To address these concerns with manure management, an integrated swine manure treatment system was proposed that includes a biological process for cleaning the water in manure and producing biomass, which is subsequently converted to bioenergy products using hydrothermal processes. A MABB was operated with and without the addition of GAC to extract CECs and other organics from the LPAM and resulting biomass, which can serve as a biofuel feedstock and fixate carbon dioxide (CO_2). According to Rossner et al., (2009), activated carbon was a more effective adsorbent for removing estrogenic hormone mixtures ($\geq$ 98%) from lake water than zeolite and ion-exchange resin due to its larger volume of pores. For example, powdered activated carbon (PAC) removed more than 90% of Bisphenol A (BPA), E2, and EE2 from drinking-water matrices (Yoon et al., 2003). Furthermore, previous studies have shown that sorption to biomass, consecutive biodegradation, and photolysis are the primary mechanisms for removing estrogenic hormones in an algal wastewater treatment system, and it was adsorbed well by various strains of algae (Shi et al., 2010; Yu et al., 2013; Zhang et al., 2014).

Several minerals such as calcium (Ca), chloride (Cl), Cu, iodine (I), iron (Fe), manganese (Mn), phosphorus (P), selenium (Se), sodium (Na) and Zn were used for pigs as feed additives by regularly adding these minerals into the diet (Morris. 1987). For example, Cu, Zn, and Fe were added in swine diets to promote growth and health (Burton. 2007). To reuse the liquid swine manure instead of disposal, the overall occurrence and distribution of heavy metals should be

analyzed in the integrated manure management system because they can potentially affect the environmental safety and human health (Nzihou & Stanmore. 2013). For example, exposure to toxic heavy metals such as As, Pb, Cu, Zn, and Cd can have carcinogenic and toxicological effects on the environment, living organism, and human beings (Brathwaite & Rabone. 1985; Zhou et al., 2015). The maximum limits of the toxic heavy metals for irrigation and livestock drinking water are as follows: As (0.1 – 0.2 mg/L), Pb (0.1 – 5 mg/L), Cu (0.2 – 0.5 mg/L), Zn (2 – 24 mg/L), and Cd (0.01 – 0.05 mg/L) (Ayers et al., 1985; U.S.EPA. 1974). If the heavy metals in inputs and outputs of bioreactors and hydrothermal bioenergy processes are characterized, the fate of heavy metals in the proposed integrated manure management system could be determined in order minimize the environmental impacts by removing residual toxic heavy metals. The final goal was to investigate the fate and transport of E1, E2, and E3 in an integrated swine manure management system that can simultaneously provide significant environmental benefits and valuable energy products. The novel manure treatment system that can capture CECs from animal manure and convert them into valuable bioenergy products instead of polluting the environment has the potential to provide a cost-effective way to reduce water pollution, enhance bioenergy production, and increase opportunities for reuse of the aqueous portion of manure.

The objectives of this study are to 1) Demonstrate the ability to extract and concentrate CECs and other organics from LPAM to create a feedstock for bioenergy production. 2) Assess the effects of GAC in MABB and CAS on the removal of CECs from LPAM and water quality parameters. 3) Characterize the chemical and biological quality of effluents from MABB and CAS under different operating conditions. Therefore, it is of great interest to better understand the fate, transport, and transformation of these antibiotics and estrogenic hormones during biological processes, and develop proper waste management processes that cost-effectively reduce the spread of these compounds in the environment. Also, developing manure-management alternatives that

mitigate these environmental impacts would be highly advantageous. Finally, MABB and thermochemical conversion of bio-waste will be combined to produce mixed algal biomass for biocrude oil, remove emerging contaminants, convert organic contaminants to bioenergy, and prepare the water for reuse. Specifically, a novel manure treatment system will be developed that can capture CECs and convert them into valuable bioenergy products.

4.2 MATERIAL AND METHODS

4.2.1 CHEMICALS AND REAGENTS

Steroid hormones such as Estrone (E1, ≥99%), 17β-estradiol (E2, ≥98%), Estriol (E3, ≥99%), and 17α-estradiol (EE2, ≥98%) were used as hormone standards which were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI). Stock solutions of E1, E2, and E3 at 0.5 mg/mL were prepared in methanol. The Solid Phase Extraction (SPE) columns, *Supelclean Envi-Carb* (500mg/6mL, No. 57094) and *Supelclean* LC-Florisil (1g/6mL, No. 57057), were purchased from Supelco (Bellefonte, PA, USA). Distilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade organic solvents such as n-hexane, acetone, acetonitrile, methanol, or dichloromethane were purchased from Fisher Scientific (Fair Lawn, NJ). Natural estrogenic hormones that were commonly detectable in swine manure as well as nearby bodies of water. The florfenicol (FF, No. F1427) and florfenicol amine standard (FA, No. 32492) were procured from Sigma-Aldrich and had a purity of 99.8% or greater. FF stock solution at 1.0 mg/mL were prepared in DI water.

4.2.2 MIXED ALGAL - BACTERIAL BIOREACTOR OPERATION

MABB and CAS were operated in a sequencing batch mode to capture CECs and other organics into biomass to use as bioenergy feedstock. LPAM was fed into the bioreactors as an influent and the total volume of each reactor was 189.3L. Hydraulic retention time (HRT) was 4 days, and the settling and reaction time were 2 and 46 hours. The aeration rate of each reactor was 6L/minute (0.03 vvm: volume of air/volume of reactor/minute) for the MABB and 11L/minute (0.06 vvm) for the CAS to make the dissolved oxygen (DO) the same for both reactors. For the MABB operation, a light bar with red and blue LED lights was used to support photosynthetic algal growth, and the maximum light intensity was 350μmoles photons/m^2/s. The temperature of MABB and CAS were 18 and 16 °C, respectively. The HRT and solid retention time (SRT) of the reactor were 4 days and 25-30 days. The uptake of dissolved organics and nutrients by microalgae and bacteria in the reactor was analyzed. The aeration flow rate was set to 6.0L/minute (0.03 vvm), which is lower than the aeration rate (1.2 vvm) for the maximum growth rate of Spirulina (Ronda et al., 2012). Other operating conditions of each reactor are listed in Table 4.1.

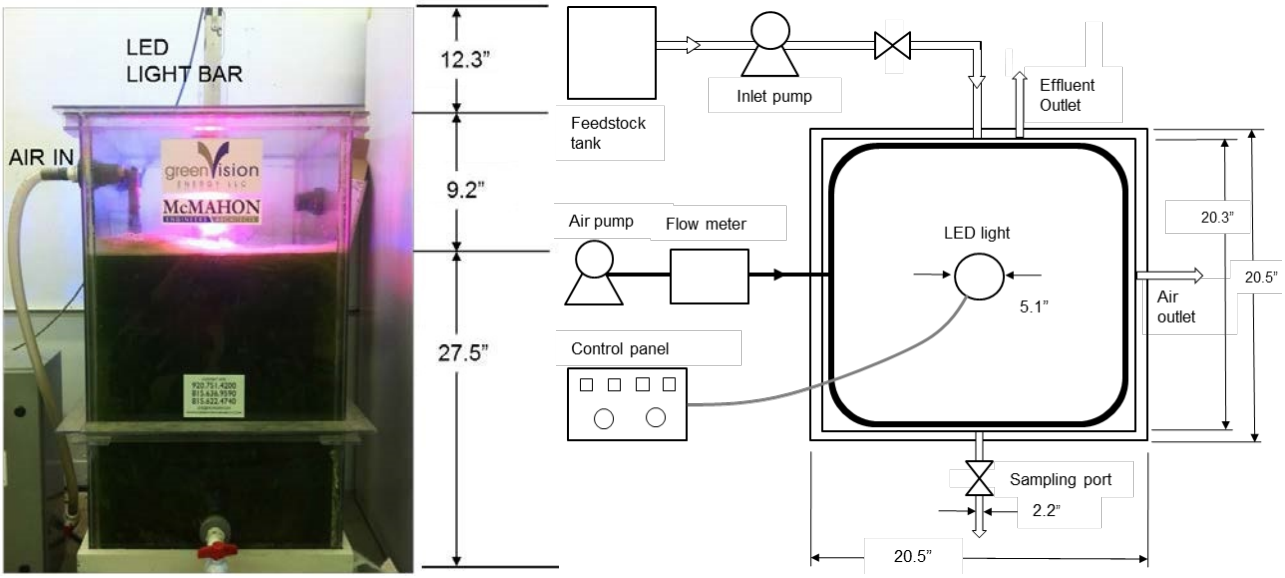


Figure 4.1 Picture and schematic diagram of mixed algal-bacterial bioreactor (MABB)

In this study, the effluent water quality parameters including the removal of CECs, dissolved organics, and heavy metals were monitored to compare the reactor performances between MABB and CAS. Also, the abilities to remove toxic compounds were compared by analyzing the cytotoxicity of the inputs and outputs from each reactor.

Table 4.1 Operating conditions for mixed algal-bacterial bioreactor

	Mixed algal-bacterial bioreactor (MABB)	Conventional activated sludge reactor (CAS)
Reactor type	Sequencing Batch Reactor	Sequencing Batch Reactor
Total Volume (L)	189.3	189.3
Light intensity (μ-photons/m^2/s)	350	-
Temperature (˚C)	18	16
Aeration rate (L/minute)	6	11
Aeration rate vvm (vessel volumes/minute)	0.03	0.06
Organic Loading Rate (mg/L/d)	46.5 – 152	46.5 - 152
HRT (day)	1- 4	1- 4
SRT (day)	25 – 30	25 - 30
Fill volume ratio (V_F/V_T, %)	50	50

4.2.2.1 GRANULAR ACTIVATED CARBON ADDITION

To investigate the effects of GAC on the ability to extract estrogenic hormones and other organics from the LPAM, treated LPAM samples from the bioreactors with and without GAC were collected to analyze the water quality, residual estrogenic hormones, heavy metals, and cytotoxicity. In this study, GAC was used because it is easily separable from the bulk liquid. Filtrasorb®400 GAC (Calgon Carbon, Pittsburgh, PA) were screened with a mesh size No. 50 at 0.297mm to achieve the desired average particle size of the GAC and washed several times using deionized water to remove dust and particles followed by drying at room temperature (AWWA

B604, 1992). The cleaned GAC were packed into mesh bags and the GAC bags were vertically installed in the bioreactors to allow the airflow to provide contact between the wastewater and GAC as the wastewater circulated. After feeding the spiked LPAM with hormones, the samples were taken regularly using glass containers for more than one time. All the samples were processed based on the procedure outlined in Chapter 3, and then the hormone concentrations in each sample were analyzed using GC/MS.

4.2.2.2 BIOMASS PRODUCTIVITY AND PROPERTIES

Table 4.2 shows experimental and theoretical calculations to analyze the biomass productivity of MABB and CAS in this study. All the measurement and calculations about biomass are total biomass quantification, heterotroph, chemoautotroph, and photoautotroph, which are the same for both reactors except photoautotroph only for MABB.

A) BIOMASS PRODUCTIVITY

The total biomass was quantified by dry cell weight, which was measured as total suspended solids (TSS) according to standard methods (APHA. 2005). To harvest biomass from each reactor, the suspended biomass in the reactor was removed every 2 days after settling for 2 hours in the sequencing batch mode. Settled biomass was harvested using the tube on the bottom of the MABB and CAS reactor, and the collected biomass was dewatered by a flat sheet Kubota membrane unit (0.4μm, cartridge type 203). The wet mixed biomass, which contains 19.3 ± 0.2 % of total solids content, was obtained by homogenizing it through a high shear mixer and kept at 4 °C in a cold room before processing. The solids content was examined by baking at 105°C in a convection oven (DKN 400, Yamato Co.) for approximately 24 hours. The volatile solid content

was examined by heating the sample at 550°C in a furnace (Barnstead Thermolyme Co.) for 3 hours or until the weight was stable. The resulting volatile fraction was 81.9 ± 0.04%.

Stoichiometric equations were used to calculate the heterotrophic biomass productivity based on the measured sCOD removal in the MABB and CAS bioreactor. According to Ebeling et al., (2006), the chemical formula of organics in wastewater can be assumed to be the same as glucose. The heterotrophic biomass yield was derived based on every gram of organic used by equations (4-1) and (4-2):

$$\underset{3(180)}{3C_6H_{12}O_6} + \underset{8(32)}{8O_2} + \underset{2(17)}{2NH_3} \rightarrow \underset{2(113)}{2C_5H_7NO_2} + 8CO_2 + 14H_2O \quad (4\text{-}1)$$

Afterwards, the biomass yield for every gram of COD is determined by:

$$\underset{(180)}{C_6H_{12}O_6} + \underset{6(32)}{6O_2} \rightarrow 6CO_2 + 6H_2O \quad (4\text{-}2)$$

To calculate the autotrophic biomass in CAS, the total harvested biomass was subtracted by the theoretical heterotrophic biomass (Table 4.2). In the MABB, both chemoautotrophs and photoautotrophs are included in the autotrophic biomass, and majority of the chemoautrotrophs are obtained by nitrifying organisms such as Nitrosomonas and Nitrobacter which oxidizes ammonia and nitrite to perform energy producing activities (Mancinelli. 1996). The following equations from (4-3) to (4-5) determines the Nitrifier biomass yield, and photoautotrophic biomass productivity in the MABB can be calculated using the equations in Table 4.2.

Nitrosomonas: $55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7NO_2 + 54NO_2^- + 57H_2O + 104H_2CO_3$ (4-3)

Nitrobacter: $44NO_2^- + NH_4^+ + 4H_2CO_3 + HCO_3^- + 195O_2 \rightarrow C_5H_7NO_2 + 3H_2O + 400NO_3^-$ (4-4)

Total: $NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.021C_5H_7NO_2 + 0.98NO_3^- + 1.041H_2O + 1.88H_2CO_3$ (4-5)

B) BIOMASS PROPERTIES

To investigate the biomass properties in each bioreactor, the biomass produced from MABB and CAS was analyzed for protein, fiber, ash, and sulfur. Biomass samples were taken from each reactor under steady – state conditions with and without GAC addition, and the samples were sent to the Midwest Laboratories, Inc. to analyze chemical components which are directly related to energy yield in the hydrothermal processes. For the analysis of biomass properties in Midwest lab, mineral analysis was performed by ICAP (Inductively coupled argon plasma) using a wet digest procedure.

Table 4.2 Summary of experimental and theoretical equations to investigate the biomass productivity in mixed algal bioreactor and conventional activated sludge reactor

Title	MABB	CAS
Total biomass quantification	Total Suspended Solid (APHA. 2005)	
	$Y_{biomass/organic}=\frac{\Delta(C_5H_7NO_2)}{\Delta(C_6H_{12}O_6)}=\frac{2(113\frac{g}{mole})}{3(180\frac{g}{mole})}=0.42\ \frac{g\ cell\ produced}{g\ organic\ used}$ (4-1)	
Heterotroph	$f_{COD}=\frac{\Delta(O_2)}{\Delta(C_6H_{12}O_6)}=\frac{6(32\frac{g}{mole})}{(180\frac{g}{mole})}=1.07\ \frac{g\ O_2\ needed\ (COD)}{g\ organic\ digested}$ (4-2)	
	$Y_{biomass/COD}=\frac{Y_{biomass/COD}}{f_{COD}}=\frac{0.42\ (\frac{g\ cell\ produced}{g\ organic\ used})}{1.07\ (\frac{g\ COD}{g\ organic\ digested})}=0.39\ \frac{g\ cell\ produced}{g\ COD}$ (4-2)	
Chemoautotroph	$Y_{nitrifier/NH4}=\frac{\Delta(C_5H_7NO_2)}{\Delta(NH_4-N)}=\frac{0.021(113\frac{g}{mole})}{(14\frac{g}{mole})}=0.16\ \frac{g\ cell\ produced}{g\ NH_4-N\ oxidized}$ (4-5)	$Y_{auto}=Y_{Total}-Y_{hetero}$
Photoautotroph	$Y_{photoauto}=Y_{Total}-Y_{hetero}-Y_{nitrifier}$	NA

4.2.3 ANALYTICAL METHOD FOR FLORFENICOL

This method is based on a procedure used by the United States Department of Agriculture (USDA) to measure FF in bovine liver and muscle tissue. The sample was digested, hydrolyzed with an acid catalyst, and converted to the amine form. The neutral lipids were then extracted with dichloromethane (DCM). The FA was converted to a free base with sodium hydroxide and

extracted from the solution with ethyl acetate. Finally, a boronate FA derivative was formed, N, N-dimethylformamide (DMF), that could be analyzed by GC/MS.

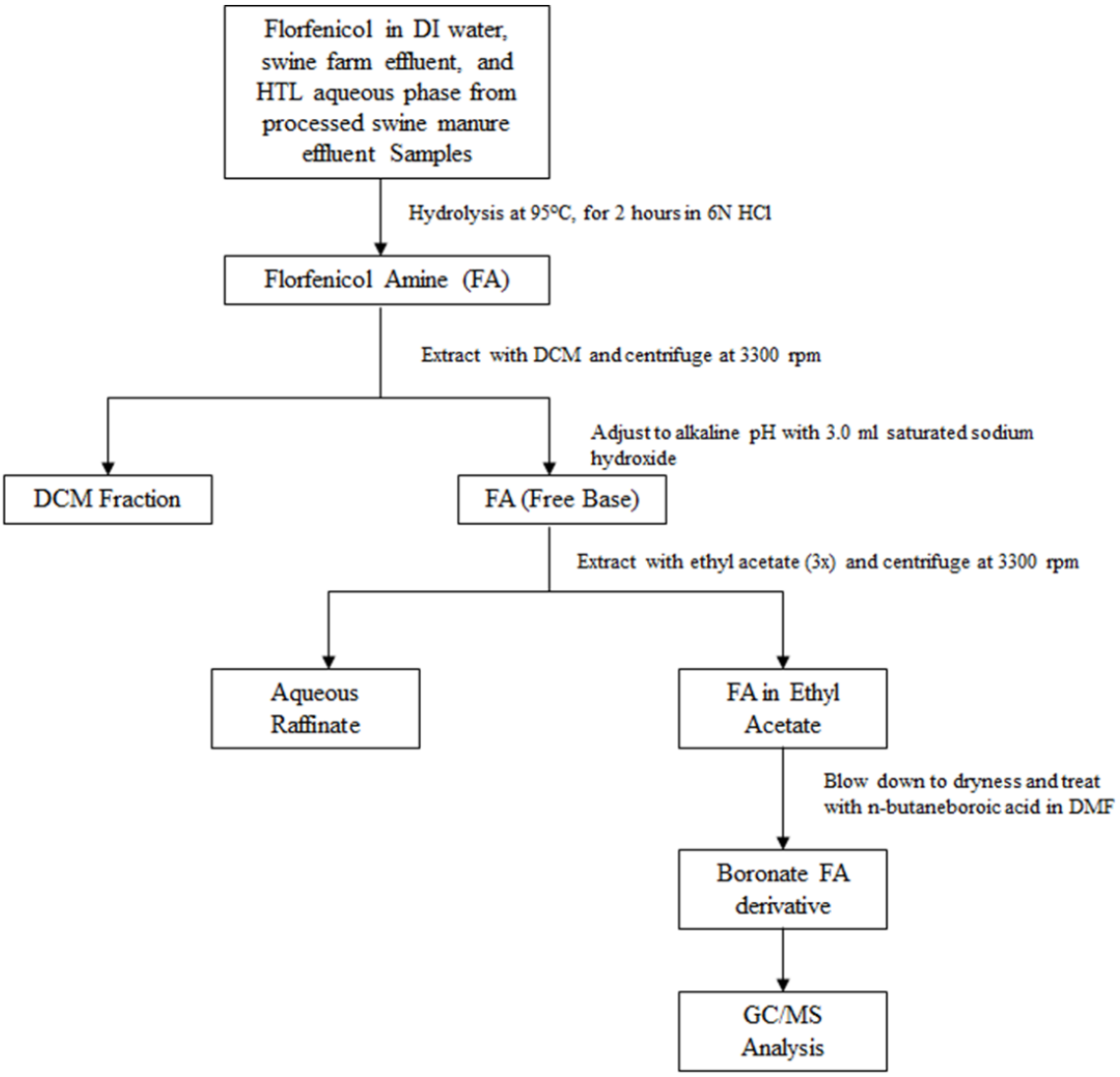


Figure 4.2 Flow diagram of the samples preparation method for florfenicol analysis by GC/MS

The USDA method used the standard GC/MS system. Our method used a high resolution one. FA stock solution in methanol was used to make calibration standards in 4.5 mL portions of ethyl acetate. The standards were then blown dry and derivatized in 1 mL mixtures of DMF and

n-butaneboronic acid (5mg/mL). The mass of the boronate FA derivative taken from a high-concentration standard was measured by the high-resolution GC/MS. This mass determination was utilized to quantify the FA in all subsequent experiments. The detection limit for FA ranged from 0.05 to 10mg/L.

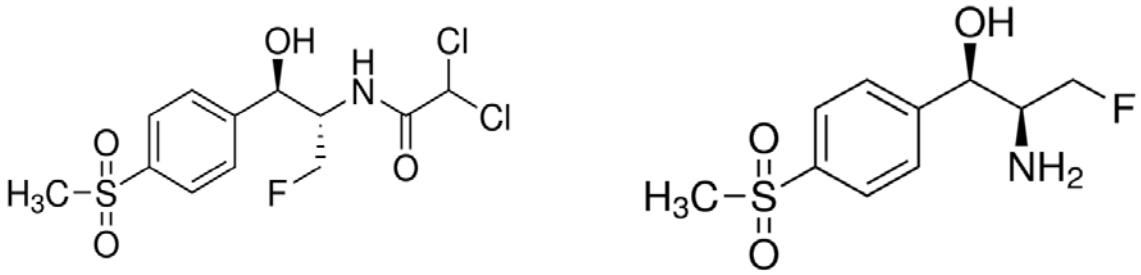


Figure 4.3 (A) Chemical structure of Florfenicol (B) and Florfenicol Amine

4.2.4 EFFECTS OF GAC ON THE FATE OF CECS

4.2.4.1 REMOVAL OF CECS DURING MIXED ALGAL AND BACTERIAL BIOREACTOR

The LPAM was spiked with hormones (3µg/L and 198.7 µg/L) and FF (330 µg/L) using a stock solution including E1, E2, E3, and FF to investigate the fate and transport of CECs in the bioreactors. After spiking the MABB and CAS with CECs, the samples were taken regularly using glass containers for one cycle of operation time. All of the samples were centrifuged at 3,500 rpm for 15 minutes to minimize Carbograph SPE column plugging by removing the suspended solids, and the residual hormones in the liquid fraction were concentrated by Solid Phase Extraction based on the procedure to eliminate the matrix effects (Hanselman et al., 2003; Singh et al., 2013; Zheng et al., 2008). The concentrates were stored at -20˚C for further analysis. The concentrations of estrogenic hormones and FF in the liquid fraction were analyzed using GC/MS as described in Chapter 3 and Chapter 4, respectively.

For a statistical analysis of the results, a student t-test was used to investigate the statistical trend of the data. The one parametric test and the two-sample Student t-test is probably the most frequently used statistical procedure for testing differences between the means of two samples. The t-test is any statistical hypothesis test in which the test statistic follows a Student's t-distribution under the null hypothesis. The P-value is the probability that the current result will occur if the correlation coefficient was in fact zero (null hypothesis). If this probability is lower than the conventional 5% ($P<0.05$) value, the correlation coefficient is deemed statistically significant (Mandel. 2006; Montgomery & Loftis. 1987). In this study, an unpaired two samples t-test with unequal variance was used to calculate the P value.

4.2.4.2 EFFECTS OF GAC ON THE FATE OF CECS

According to Rossner et al., (2009), activated carbon is a more effective adsorbent for removing estrogenic hormone mixtures (≥ 98%) from lake water than zeolite and ion-exchange resin, due to its larger volume of pores. In addition, particular activated carbon (PAC) removed more than 90% of BPA, E2, and EE2 from drinking-water matrices (Yoon et al., 2003). Furthermore, previous studies have shown that sorption is the major mechanism for removing estrogenic hormones including E1 in an algal-based wastewater treatment system, and it was absorbed well by various strains of algae (Shi et al., 2010). Also, activated carbon (AC) was the most effective adsorbent for extracting hormones from the aqueous phase compared to adsorbents such as zeolite and ion-exchange resin (Rossner et al., 2009). In this test, GAC was used due to the ease of separating it from the bulk liquid. The enhancing effects of GAC on the extraction of hormones and organics from LPAM to create feedstock for bioenergy production were investigated in MABB and CAS. The hormones in the treated LPAM were analyzed based on the

method Chapter 3, and water quality parameters were monitored to investigate the effects of GAC on the performance of the bioreactors under steady-state operation.

4.2.5 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF INPUTS AND OUTPUTS

4.2.5.1 WATER QUALITY ANALYSIS

Soluble chemical oxygen demand (sCOD), total dissolved nitrogen (TDN), ammonia nitrogen (NH_3-N), and total dissolved phosphorus (TDP) of the water samples were analyzed as described in the previous chapter. In addition, the pH and dissolved oxygen (DO) of each reactor were monitored using a pH sensor (pH-BTA, Vernier Software & Technolgy, OR, USA) and DO probe (DO-BTA, Vernier Software & Technolgy, OR, USA) with a real-time data collection and analysis device (Labquest 2, Vernier Software & Technolgy, OR, USA).

4.2.5.2 HEAVY METAL ANALYSIS

Samples that were clear and contained no particulates were filtered through a 0.45µm filter prior to analysis. For solid and aqueous samples containing particulate, a sub-sample was microwave digested into solution by the United States Environmental Protection Agency (U.S. EPA) Method 3051A (U.S. EPA 2007a). Chromium (Cr), Mn, Zn, As, rubidium (Ru), molybdenum (Mo), Ca, and Pb measurements were performed with a VG Elemental PQ Excel ICP-MS per U.S. EPA Method 6020A (U.S. EPA, 1998). Na, Ca, potassium (K), and Fe measurements were performed with a Varian Spectra AA 55B atomic absorption instrument per U.S. EPA Method 7000B (U.S. EPA 2007b). Both instruments were calibrated using reference materials procured from SpexCertiprep and internal standards were used during the ICP-MS analysis. In addition, a certified reference material (Dogfish Liver Certified Material for Trace Metals, DOLT-3), a duplicate sample, and an analytical sample spike were performed during both

assays as quality control parameters. The results of the reagent blank indicate no contamination was introduced during sample preparations.

4.2.5.3 CHO CELL MAMMALIAN CYTOTOXICITY

CHO cell mammalian cytotoxicity of inputs and outputs from MABB and CAS bioreactor were investigated depending on the method in Chapter 3. However, the samples were not extracted using XAD resins rather the LPAM, MABB and CAS aqueous samples were used to make a sample F2 + FBS medium. Each aqueous sample was used to prepare the F12 + 5% FBS sample medium. A known volume of sample was mixed with F12 medium power, the pH was adjusted to 7.4 using $NaHCO_3$ and the solution was filter sterilized over a 0.22 µm filter. Sterile FBS was added to the sample medium to a concentration of 5%. A 96-well cell culture microplate was used, with a series of concentrations of the sample medium mixed with the cell culture F12 + FBS culture medium. Each microplate well contained an independent clone of cells and representing an independent measurement. Eight wells on the microplate a served as the negative controls comprising the F12 + FBS medium and 3×10^3 CHO cells, and eight other wells on the same plate served as the blank controls comprising only the F12 + FBS medium (Dong et al., 2016). The remaining wells contained a known concentration of a sample medium, F12 +FBS medium, and 3×10^3 CHO cells for a total volume of 200 µL. The specific organic concentrations for chronic cytotoxicity tests were determined based on the sCOD results of each sample (Pham. 2013).

To analyze mammalian cell cytotoxicity, reduction in cell density on flat-bottom tissue culture 96-well microplates was measured as a function of a wastewater sample over a period of approximately 3-4 cell divisions (72h) (Plewa et al., 2009; Plewa et al., 2010; Wagner & Plewa. 2017). Four replicate wells were prepared for each concentration of a specific organic extract for each sample. The experiments were repeated at least 2 times, and a regression analysis was

conducted for each concentration-response curve. The LC_{50} value is the concentration of the wastewater sample that induced a cell density of 50% as compared to the concurrent negative controls. To determine if there was significant difference in cytotoxicity among the individual samples, the mean cytotoxic index (CTI: $LC_{50}^{-1}\times10^{3}$) and its standard error were calculated for each LC_{50} value, and evaluated by an analysis of variance (ANOVA) pairwise test.

4.2.5.4 ACUTE TOXICITY

According to the method Chapter 3, the acute toxicity in the samples from MABB and CAS were analyzed to compare with CHO cell cytotoxicity of the samples. The specific organic concentrations for microtox bacterial toxicity tests were also determined based on the sCOD results of each sample.

4.2.5.5 YES YEAST CELL ASSAY FOR ESTROGENIC ACTIVITY

Chapter 3 describes a method to investigate the estrogenic activity of effluents from MABB and CAS. SPE cartridges were used to extract the samples, and DMSO was used to elute the extracts to use in a XenoScreen YES assay to characterize test compounds in the samples with activating (agonistic) activities. For the YES assay, based on the analysis of estrogenic hormones in addition to E2 concentrations of each sample, specific E2 equivalent concentrations were determined. EEQ expressed the estrogenic potency of a compound or environmental extract. Usually, a compound's estrogenic potency was expressed relative to E2 by EC_{50} values, where the highest estrogenic effect was observed 50% of the time at this concentration.

4.2.5.6 ANTIBIOTIC RESISTANCE ASSAY

To investigate the possible biological effects of antibiotics in livestock manure, a fluctuation test was developed to measure the antibiotic resistance in bacterial populations exposed to antibiotics (Pals & Plewa. 2015). For the initial proof of concept, *E. coli* 15597 was exposed to Kanamycin at a concentration that provides a selective advantage to individual bacterium in the population. Because the antibiotic can kill or inactivate cells which lack the favorable genotype, cells with resistant genotypes, have a reproductive advantage and gradually dominate the population. *E. coli* grown to the stationary phase in the Luria-Bertani (LB) medium with non-lethal antibiotics (2mg/L) became resistant to normally lethal concentrations of antibiotics (17mg/L). The "selective concentration" was a concentration of antibiotic that reduced the growth rate, measured as a change in optical density at a wavelength of 600nm (OD_{600}) of an initial inoculation of *E. coli* in LB medium + antibiotic as compared to the same number of bacteria inoculated in LB only, and the "lethal" concentration was a concentration of antibiotic in LB that eliminated growth. The results of this experiment showed that bacteria grown in 3 consecutive overnight cultures of LB + 2mg/L antibiotic were more likely to produce growth in lethal (17mg/L) concentrations of antibiotic. This test was applied to LPAM to define the baseline of antibiotic resistance. Subsequently, this test was applied to treated LPAM, processed by the MABB to define the effect of this bioprocess on the potential of LPAM to foster the development of antibiotic resistance. The positive and negative controls of the MABB effluent were compared to identify the antibiotic resistance generated by FF.

4.3 RESULTS AND DISCUSSIONS

4.3.1 EFFECTS OF GAC ON THE ALGAL BIOREACTOR OPERATION

4.3.1.1 WATER QUALITY ANALYSIS

Table 4.3 depicts a summary table for the effluent water quality and nutrient removal of the MABB and CAS, which are related to the ability to extract organics and nutrients from the LPAM. Also, these results described the benefits and effects of adding GAC to the performance of bioreactors for biomass productivity.

A) SCOD REMOVAL

Table 4.3 and Figure 4.4 (A) shows the influent and effluent sCOD concentrations from the MABB and CAS reactors with and without GAC. The average effluent sCOD concentration for the MABB and CAS processes with and without GAC were 45.7 ± 1.3 and 63.1 ± 2.2, 48.3 ± 2.3 and 66.2 ± 3.2mg/L, respectively. The percent removal of sCOD in the effluent from MABB with and without GAC were 74.6 ± 0.9 and 66.2 ± 1.6%, which are slightly higher than the results from CAS (73.4 ± 1.7 and 64.6 ± 2.3%). These results suggest that MABB and CAS showed similar abilities in removing dissolved organics, even though the aeration rate of MABB (6.0L/minute) was lower than that of CAS (11L/minute) to make the dissolved oxygen (DO) the same for both reactor. These results proved that MABB was more energy effective than CAS because algae could contribute to enhancing the bacterial growth by providing photosynthetic oxygen which is one of the benefits stemming from their symbiotic relationship (Fuentes et al., 2016; Ramanan et al., 2016).

Table 4.3 Summary of steady-state removal of priority pollutants in a mixed algal and conventional activated sludge reactor with and without GAC

	MABB		CAS	
	W/ GAC	W/O GAC	W/ GAC	W/O GAC
Average Dissolved Oxygen (mg/L)	7.2 ± 0.3	7.1 ± 0.3	7.5 ± 0.3	7.3 ± 0.4
Average pH	7.6 ± 0.3	7.2 ± 0.2	7.8 ± 0.4	7.5 ± 0.3
Effluent water quality (mg/L)				
SCOD	45.7 ± 1.3	63.1 ± 2.2	48.3 ± 2.3	66.2 ± 3.2
TDN	150.7 ± 0.6	157.4 ± 6.2	144.9 ± 2.9	154.9 ± 1.4
NH3-N	1.75 ± 0.4	4.0 ± 0.4	1.84 ± 0.6	3.2 ± 0.1
TDP	17.9 ± 0.1	19.9 ± 0.3	20.5 ± 0.5	22.01 ± 0.2
Pollutant removal percentage (%)				
SCOD	74.6 ± 0.9	66.2 ± 1.6	73.4 ± 1.7	64.6 ± 2.3
TDN	30.1 ± 0.3	24.0 ± 4.2	33.1 ± 0.9	23.7 ± 1.4
NH3-N	97.0 ± 0.1	93.5± 0.7	97.3 ± 0.1	94.9 ± 0.2
TDP	39.5 ± 1.0	31.4 ± 0.5	30.8 ± 1.8	23.6 ± 0.3
Pollutant removal rate (mg/L-d)				
SCOD	67.9 ± 0.3	61.9 ± 0.2	66.7 ± 0.6	60.4 ± 0.8
TDN	32.5 ± 0.2	25.0 ± 1.6	33.0 ± 0.7	23.7 ± 0.4
NH3-N	30.8 ± 0.1	29.2 ± 0.2	30.7 ± 0.1	29.7 ± 0.1
TDP	6.1 ± 0.3	4.9 ± 0.1	4.8 ± 0.1	3.8 ± 0.2

According to Min et al., (2014), liquid swine wastewater contains a significant amount of benzene ring structures derived from lignin, which can explain the relatively low biodegradability. After removing the GAC from each bioreactor, percent removal of sCOD in the effluents of the MABB and CAS reactors decreased up to 8.4 and 8.8%, respectively. These results generally agree with previous reports on the recalcitrant organic fraction in swine wastewater (Lee et al., 2008). GAC was effective in removing many the biologically recalcitrant compounds in the wastewater treatment process, which can also inhibit biological degradation processes. Thus, GAC resulted in the improvement of both effluent organic concentrations.

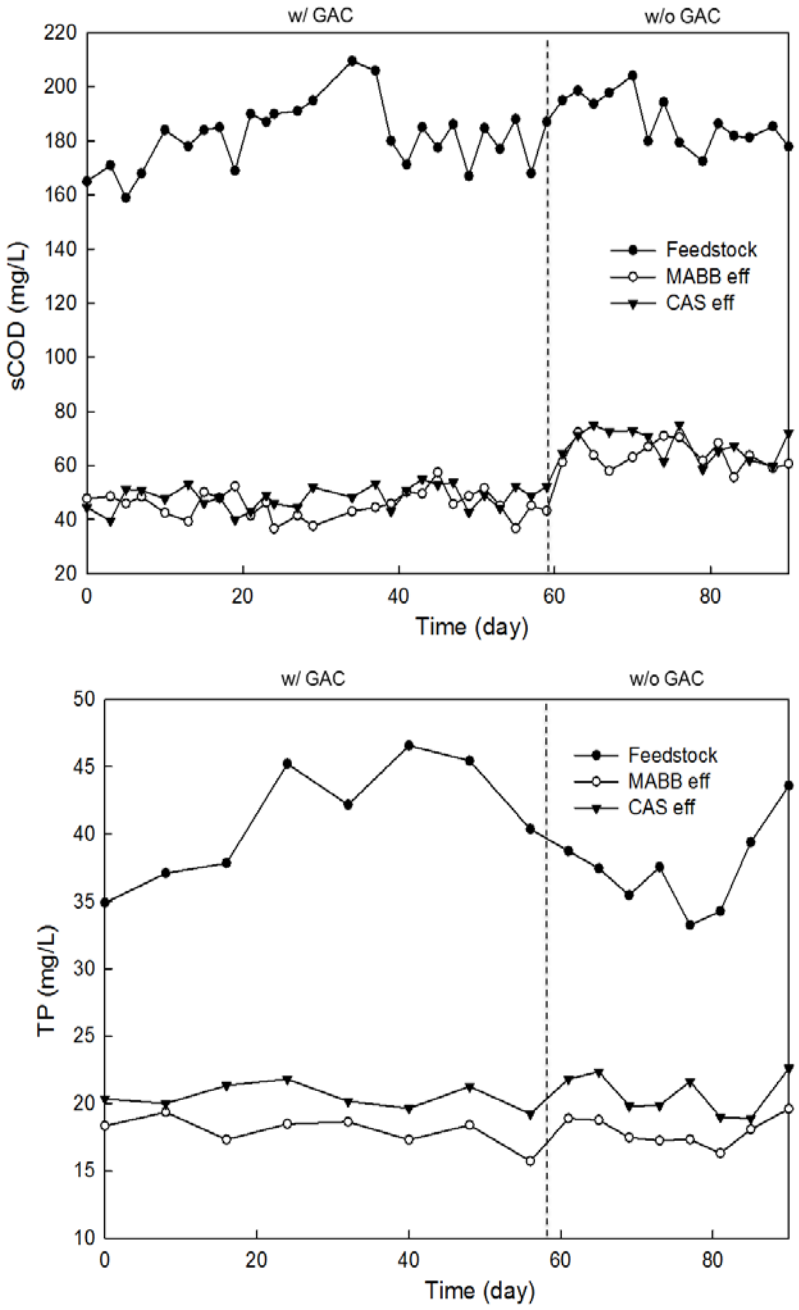


Figure 4.4 (A) Effects of GAC on the removal of dissolved organics (B) Effects of GAC on the removal of total phosphorus.

B) PHOSPHORUS REMOVAL

As shown in Table 4.3 and Figure 4.4 (B), the percent removal of TDP in MABB with and without GAC, and CAS with and without GAC were 39.5 ± 1.0, 31.4 ± 0.5, 30.8 ± 1.8, and 23.6 ± 0.3%, respectively, which demonstrated that MABB had a better phosphorus removal rate than CAS for LPAM. According to Roeselers et al., (2008) and Larsdotter, (2006), the main removal mechanisms of phosphorus were biological assimilation and chemical precipitation. Thus, Kromkamp, (1987) proved that cyanobacteria could store it internally as polyphosphates, which could support the notion that MABB had a higher removal rate of total phosphorus than CAS. After removing the GAC, the percent removals of TDP were decreased up to 8.1 and 7.2 % in MABB and CAS, respectively. The addition of GAC into bioreactors enhanced the removal of total phosphorus, which supported the idea that the chemical adsorption of phosphate onto activated carbon could be occurred and contributed to increase the percent removal of total phosphorus from 66.2 ± 1.6 to 74.6 ± 0.9% and from 64.6 ± 2.3 to 73.4 ± 1.7% in MABB and CAS, respectively (Zhang et al., 2011). The Illinois EPA is currently issuing wastewater discharge permit renewals that require all water discharged into a river or lake to contain less than 1 mg/L of phosphorous as P (IPAC, 2014), and all the effluents from bioreactors did not meet the discharge standard, even though GAC was used to enhance the removal of phosphorus. Therefore, additional treatment methods or adsorbents are necessary to further treat the LPAM to meet discharge regulations.

C) NITROGEN REMOVAL

In the Table 4.3 and Figure 4.5 (A), the average TDN percent removal in the effluents from MABB with and without GAC, as well as CAS with and without GAC were 30.1 ± 0.3, 24.0 ± 4.2, 33.1 ± 0.9, and 23.7 ± 1.4%, respectively. These results demonstrate that CAS is slightly better in

removing TDN than MABB from LPAM. Adding GAC enhanced the removal of TDN by 6.1 and 9.4% in MABB and CAS. Furthermore, the TDN removal rates of MABB with and without GAC and, CAS with and without GAC were 32.5 ± 5.7, 25.0 ± 1.6, 33.0 ± 0.7, and 23.7 ± 0.4mg/L-d, respectively, which are slightly lower than the results from previous studies (MABB: 38.3 – 46.38mg/L/d; CAS: 42.3 – 49.3mg/L/d) under similar applications (Min et al., 2014; Park et al., 2011). Because the system was operated aerobically, which limited denitrification, and because ammonia volatilization and precipitation is minimal at neutral pH levels observed in this study (Liao et al., 1993; Nelson et al., 2003), the reduction of TDN was mostly caused by biological assimilation.

As shown in Table 4.3 and Figure 4.5 (B), ammonia concentrations in the effluents from MABB with and without GAC, CAS with and without GAC were 1.75 ± 0.4, 4.0 ± 0.4, 1.84 ± 0.6 and 3.2 ± 0.1mg/L, respectively, which means that all the effluents except MABB with GAC and CAS with GAC did not met the discharge standard because the Illinois EPA requires that all water discharged into rivers or lakes contain less than 2.5mg/L of ammonia nitrogen (IPAC, 2014). These results demonstrated that the MABB and CAS had similar abilities in removing ammonia from the LPAM, and the percent removal of NH_3-N was increased up to 3.67 and 2.31% in MABB and CAS after adding GAC because GAC was effective in removing recalcitrant compounds that can inhibit the growth of nitrifiers in each reactor (Ji et al., 2015; Serrano et al., 2013; Zhao et al., 2013).

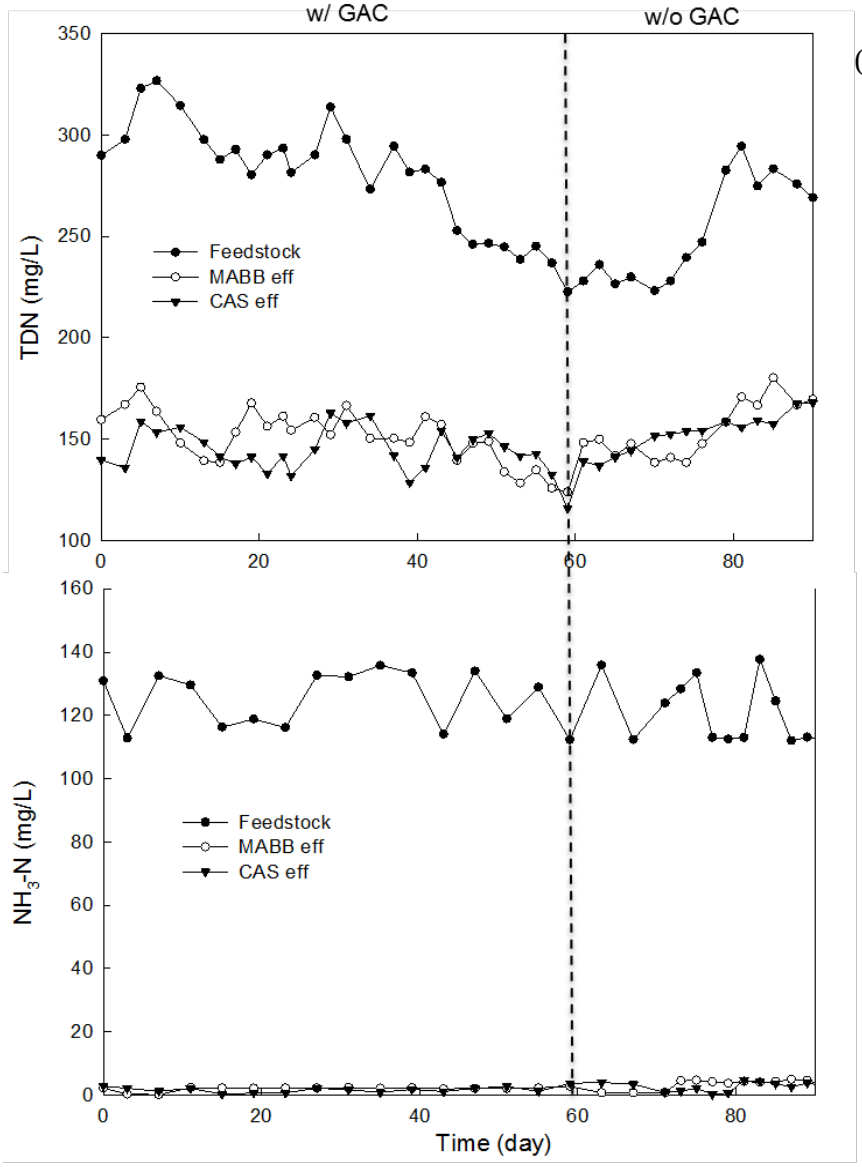


Figure 4.5 (A) Effects of GAC on the removal of total dissolved nitrogen (B) Effects of GAC on the removal of ammonia nitrogen.

D) DISSOLVED OXYGEN

The air flowrate was adjusted to enhance the energy efficiency of bioreactor operation by monitoring the dissolved oxygen (DO) in each reactor because aeration is the dominant energy consuming part in the system, which was 44% - 50% of the total energy consumption in typical activated sludge systems (Lazić et al., 2012; SevernTrentWater. 2014). Considering the reactor was mixed using the up-flow aeration, 5.0L/minute (0.026vvm) was the minimum air flowrate to stir up the suspended biomass in each bioreactor. DO values of each system were equalized to 6.8mg/L on average by using an air flowrate of 6.0L/minute for MABB and 11.0L/minute for CAS, and DO was ranged from 0.8 to 7.6mg/L in one cycle of a sequencing batch operation including 2 hours of sedimentation process without aeration. To study the effects of aeration in two different types of bioreactors, water quality parameters such as sCOD, TN, TP, and NH_3-N were monitored for each cycle of the batch test. As shown in Table 4.3, the percent removal of sCOD, TDN, TDP, and NH_3-N in MABB/CAS were 74.6/73.4%, 30.1/33.1%, 39.5/30.8%, and 97/97.3%, which demonstrated that the two reactors had similar performance results with different aeration rates. Based on typical algal photosynthesis stoichiometry, 1.34 gram of oxygen was generated per gram of algal biomass synthesized (Brune et al., 2003). The productivity of photoautotrophs in the MABB with GAC was 10.4mg/L-d, which equals to 13.9mg/L-d oxygen generated in water, and it is lower than in previous studies where the average algal biomass productivity of well operated high-rate algal pond was 44mg/L-d and produced 58.6mg/L-d oxygen in water for organic and ammonia oxidation (Lundquist et al., 2010). Therefore, MABB was more energy effective than CAS because the produced oxygen from algae in MABB could increase DO levels and lower energy consumption for aeration.

4.3.1.2 BIOMASS PRODUCTIVITY AND PROPERTIES

Table 4.4 showed the summary of biomass productivity and properties of MABB and CAS with and without GAC addition. The biomass productivity of MABB (41.8 ± 0.7mg/L/d) was higher than CAS (31.1 ± 0.1mg/L/d), and the productivity was decreased up to 23% and 8.7% in MABB and CAS after removing GAC, respectively, which confirms that the GAC can improve biomass productivity. Thus, it was proved that GAC can enhance certain water quality parameters because GAC had a better TN removal percentage than bioreactors without GAC. Since there is little to no denitrification under aerobic conditions, TN removal is mostly due to the extra biomass growth and storage of nitrogen in the biomass. In comparison to other research, integrating adsorbents could achieve higher removal rates of COD and TN as well as higher biomass productivity for both MABB and CAS.

These results showed that GAC could improve the performance of bioreactors by removing recalcitrant compounds that could inhibit biomass growth (Ji et al., 2015; Serrano et al., 2013; Zhao et al., 2013). The biomass composition with GAC was analyzed in Table 4.3 and the results demonstrated that crude proteins and carbohydrates were increased only in CAS with GAC. However, ash contents were increased in MABB (8%) and CAS (7.8%) after removing the GAC. Because ash is not useful for bioenergy production, GAC could enhance bioenergy yield in the hydrothermal processes by improving biomass properties. (Biller et al., 2012).

Table 4.4 Effects of GAC on the biomass productivity and properties in a mixed algal-bacterial bioreactor and conventional activated sludge system

	MABB		CAS	
	W/ GAC	**W/O GAC**	**W/ GAC**	**W/O GAC**
Total Biomass Productivity (mg/L-d)	41.8 ± 0.7	32.2 ± 0.4	31.1 ± 0.1	28.4 ± 0.3
Heterotrophic Productivity (mg/L-d)	26.5 ± 0.1	24.1 ± 0.1	26.0 ± 0.2	23.6 ± 0.3
Chemoautotrophic Productivity (mg/L-d)	4.9 ± 0.02	4.7 ± 0.1	4.9 ± 0.04	4.7 ± 0.02
Photoautotrophic Productivity (mg/L-d)	10.4 ± 0.6	3.3 ± 0.2	-	-
Crude Fat (%VS)	n.d.	0.72	0.67	n.d.
Crude Protein (%VS)	43.8	44.7	50.5	44
Carbohydrate (hemi-, cellulose, lignin) (%VS)	56.2	54.58	48.83	56
Ash Content (%)	19.9	27.9	19.2	27

4.3.2 EFFECTS OF GAC ON THE FATE OF CECS

4.3.2.1 REMOVAL OF ESTROGENIC HORMONES WITH AND WITHOUT GAC ADDITION

The LPAM was fed into the MABB and CAS to remove the estrogenic hormones and produce biomass for bioenergy production. The concentrations of E1, E2, E3, and EE2 in the feedstock and the effluent of each bioreactor were analyzed to calculate the percent removal of each hormone before and after the bioreactor operations. According to previous studies, the dominant removal mechanisms of estrogenic hormones in the bioreactors were sorption to biomass and subsequent biodegradation because hormones were hydrophobic compounds and a lag phase between 5 hours to 15 days for algae or bacteria to perform biological degradation in the bioreactor was present (Muller et al., 2010; Yang et al., 2010; Zhang et al., 2014). To quantify the effects of GAC on the removal of estrogenic hormones, the percent removal of estrogenic hormones was investigated for each bioreactor under various operating conditions, including the addition of

GAC. The results in Table 4.5 showed that the average percent removal of total hormones in MABB and CAS with and without GAC were as follows:

MABB w/ GAC (97.0 ± 0.6%) > CAS w/ GAC (95.3 ± 1.2%) > MABB w/o GAC (89.8 ± 1.12%) > CAS w/o GAC (81.0 ± 1.8%)

These results demonstrated that, on average, more than 85.5% of total estrogenic hormones could be removed in the bioreactors, and MABB was more effective at total hormones removal than CAS, especially without the addition of GAC because the difference in the percent removal between MABB and CAS with GAC (P=0.91) was insignificant compared to removal without GAC (P=0.32). This difference can be explained by slightly higher temperatures and the photochemical degradation by the LED lights in the MABB, which can contribute to the removal of hormones (He et al., 2012; Lin & Reinhard. 2005; Puma et al., 2010; Whidbey et al., 2012).

Table 4.5 Removal of estrogenic hormones under different operating conditions of MABB and CAS

	MABB		CAS	
	W/ GAC	W/O GAC	W/ GAC	W/O GAC
Average hormones removal (%)				
Estrone (E1)	96.4 ± 0.9	95.0 ± 2.1	94.9 ± 1.2	90.9 ± 1.0
17β-estradiol (E2)	98.2 ± 0.1	93.9 ± 0.5	97.4 ± 0.6	85.2 ± 0.6
Estriol (E3)	95.6 ± 0.9	88.7 ± 2.1	96.9 ± 0.5	81.4 ± 3.0
17α-estradiol (EE2)	91.4 ± 1.2	89.2 ± 2.8	91.4 ± 1.1	87.6 ± 2.0
Total (E1+E2+E3+EE2)	97.02 ± 0.7	89.8 ± 1.0	95.3 ± 1.2	81.0 ± 1.8
Hormones removal at low spiking (%)				
Estrone (E1)	91.3 ± 1.0	98.0 ± 0.4	94.9 ± 2.0	91.0 ± 4.4
17β-estradiol (E2)	97.6 ± 1.0	89.0 ± 3.5	92.6 ± 3.0	84.3 ± 2.2
Estriol (E3)	92.5 ± 0.5	78.8 ± 2.9	95.6 ± 0.6	82.7 ± 7.8
17α-estradiol (EE2)	87.8 ± 1.3	84.9 ± 4.4	93.7 ± 0.9	95.2 ± 0.5
Total (E1+E2+E3+EE2)	95.0 ± 0.3	85.8 ± 0.2	92.2 ± 1.4	85.5 ± 2.4
Hormones removal at high spiking (%)				
Estrone (E1)	99.8 ± 3.4	97.7 ± 0.5	98.3 ± 0.1	96.8 ± 0.8
17β-estradiol (E2)	99.1 ± 0.2	94.6 ± 1.0	99.9 ± 0.2	86.8 ± 3.6
Estriol (E3)	99.3 ± 0.4	95.4 ± 1.1	98.3 ± 0.1	80.6 ± 3.9
17α-estradiol (EE2)	96.8 ± 0.3	97.7 ± 0.6	86.7 ± 3.9	68.6 ± 3.3
Total (E1+E2+E3+EE2)	99.0 ± 0.2	92.4 ± 1.7	99.0 ± 0.5	86.9 ± 2.7

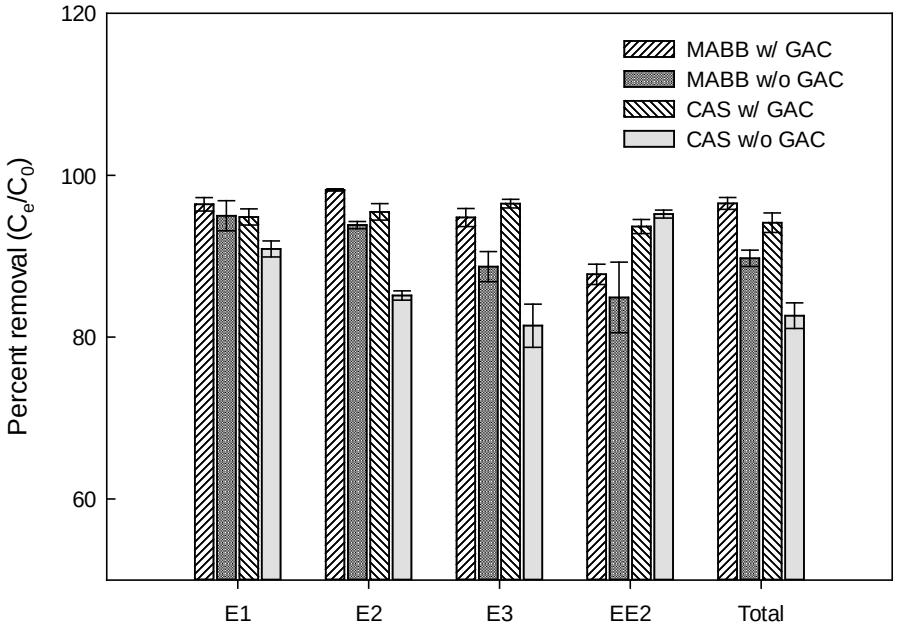


Figure 4.6 Percent removal of estrogenic hormones in the MABB and CAS under the condition of w/ and w/o GAC. The error bars indicate the standard error of the mean

In addition, Figure 4.6 also showed that GAC increased the removal of total hormones up to 8 % and 11 % in MABB and CAS, respectively, due to additional adsorption of hormones to GAC (Westerhoff et al., 2005; Yoon et al., 2003). These results demonstrated that GAC addition could enhance the removal of total estrogenic hormones in the bioreactors, and the percent removal of hormones were higher in CAS than MABB with GAC addition. Thus, the removals of E2, E3, and EE2 were more sensitive to the addition of GAC than E1 in each reactor because the increased percent removal of E2 and E3 in the bioreactors were up to 2 times higher than that of E1 (Table 4.5).

4.3.2.2 HORMONES EXTRACTION FROM BIOMASS

Figure 4.7 displays the concentrations of extracted hormones from MABB and CAS biomass using serial extraction, and the MABB biomass contains 304 ± 15µg/L more than the CAS biomass in total. The percent distribution of E1, E2, E3, and EE2 in the extracted hormones from MABB were 40: 37: 21: 2%, respectively. However, E2 was the dominant hormone in the extracted hormones from CAS biomass, and the percent distribution of E2 was 91%.

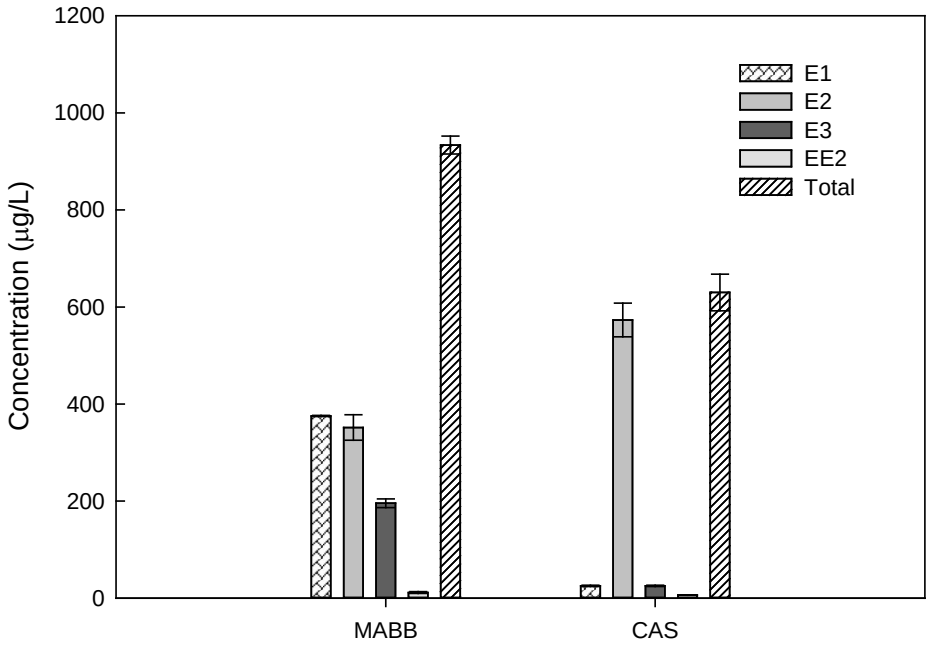


Figure 4.7 Concentrations of estrogenic hormones in the MABB and CAS biomass. The error bars indicate the standard error of the mean

4.3.2.3 REMOVAL OF FLORFENICOL WITH AND WITHOUT GAC ADDITION

The removal and distribution of the FF breakdown products (FF-BP) after biological processes were investigated when spiked LPAM with FF was fed into the MABB and CAS. Similar to the before and after hormone analysis of bioreactor operations, percent removal and distribution were determined based on the same method. Previous studies stated that the dominant

removal mechanisms of FF were photolysis, activated by the LED lights, and biological degradation which occurred in the algae bioreactor with GAC (Mitchell et al., 2013; Zhang et al., 2016a; Zhang et al., 2016b). Thus, because FF had low log Kow (- 0.04) and Koc (24−52L/kg) values, which were considered a weakly hydrophobic organic compound, it could be removed by sorption to GAC or biomass. For each bioreactor under different operating conditions, the percent removal of FF was investigated to quantify the effects of biological degradation and GAC on FF removal. Figure 4.8 and Table 4.6 showed the average FF percent removal values in MABB and CAS with and without GAC:

MABB w/ GAC (96.0) > CAS w/ GAC (95.6) > MABB w/o GAC (93.4) > CAS w/o GAC (91.3%)

The results showed that more than 91.3% of the total FF on average could be removed in the bioreactors, and CAS showed lower FF removal than MABB especially without GAC addition. However, because both reactors, MABB and CAS, with GAC showed insignificant maximum differences in percent removal (P=0.12), which could not reject the null hypothesis.

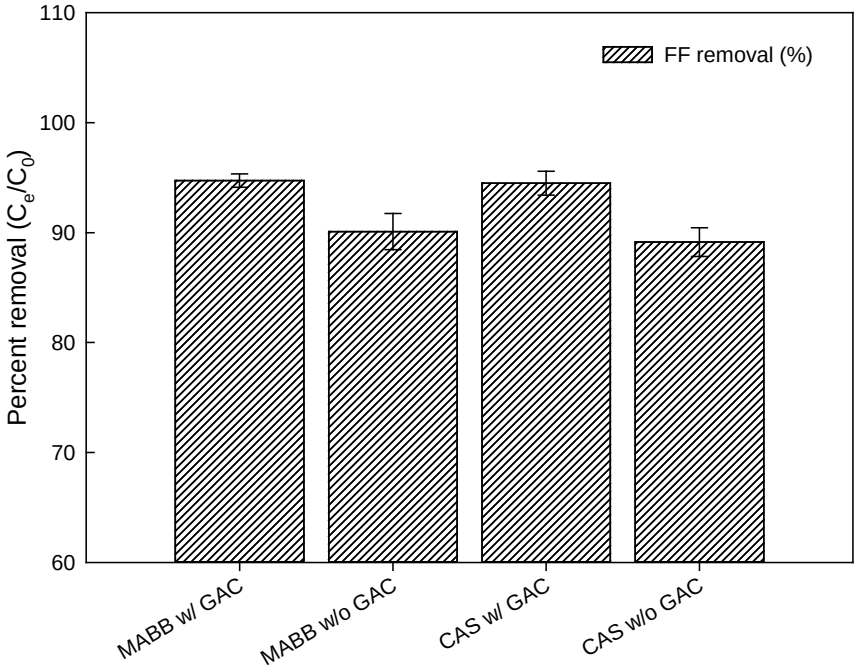


Figure 4.8 Percent removal of FF in the MABB and CAS under the condition of w/ and w/o GAC. The error bars indicate the standard error of the mean

Additionally, Figure 4.8 also demonstrated an increase in total hormone removal by GAC from 93.4 to 96.0% and 91.3 to 95.6% in MABB and CAS, respectively, because 98.5-99.2% of FF was removed using powdered activated carbon (PAC) which accounts for the additional adsorption of FF to GAC in a recent study (Zhang et al., 2016a). Therefore, enhancing the FF removal in the bioreactors is possible through GAC addition. Thus, the addition of GAC rather than MPS affected 4-MSB and 4-MSAP removal in each reactor because the subsequent distributions of 4-MSB and 4-MSAP to effluents from each bioreactor were increased from 2.1 to 5.6% and from 10.4 to 26.9% after GAC addition (Table 4.6).

Table 4.6 Removal of FF and its breakdown products under different operating conditions of MABB and CAS

	MABB		CAS	
	W/ GAC	W/O GAC	W/ GAC	W/O GAC
Average florfenicol removal (%)				
Florfenicol (FF)	96.0 ± 0.5	93.4 ± 1.5	95.6 ± 0.9	91.3 ± 0.9
Distribution of FF breakdown products to effluent (%)				
Methyl phenyl sulfone (MPS)	0.6	0.2	4.7	4.8
4-(methylsulfonlyl)benzaldehyde (4-MSB)	5.3	2.1	5.8	2.0
4'-(methylsulfonyl)acetophenone (4-MSAP)	25.1	10.5	28.6	10.3

4.3.3 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF INPUTS AND OUTPUTS

4.3.3.1 HEAVY METAL ANALYSIS

Gai et al., (2015) reported that increased risks of phytotoxicity and contamination of surface and ground water were possible as adverse effects of high metals concentrations in the environment. Thus, the concentrations of heavy metals in the inputs and outputs of MABB and CAS processes both with and without GAC addition were analyzed. The results are presented in Figure 4.9 and Figure 4.10 which show the distribution of heavy metals during the biological processes. The average percent removal of total heavy metals from LPAM in the MABB and CAS were 11.1% and 13.7%. This meant that MABB and CAS showed similar performances in removing metal compounds from LPAM under the same operating conditions (P=0.16). However, the average concentrations of toxic heavy metals (As, Pb, Cu, Zn, and Cd) in the effluents from MABB and CAS were ranged from 0.01 to 0.8mg/L and from 0.002 to 0.01mg/L, respectively,

which were significantly lower than the recommended maximum concentrations for water reuse (Ayers et al., 1985).

After adding GAC to MABB and CAS, the average percent removal of heavy metals from LPAM were increased from 2.6 to 19.6% for MABB and 4.4 to 23.0% for CAS, respectively, because of metal compounds adsorbing to GAC (Al-Omair & El-Sharkawy. 2007; Kadirvelu et al., 2001). Furthermore, the average percent distribution of heavy metals to the biomass were increased from 2.6 to 6.1% and from 4.4 to 8.5% in MABB and CAS, respectively, after adding GAC. These results showed that GAC enhanced the percent removal of heavy metals from LPAM because Pb, Cd, Ni, Cr, and Zn have been previously demonstrated to be removed by adsorption onto the activated carbon (Kadirvelu et al., 2001; Udeye. 2009).

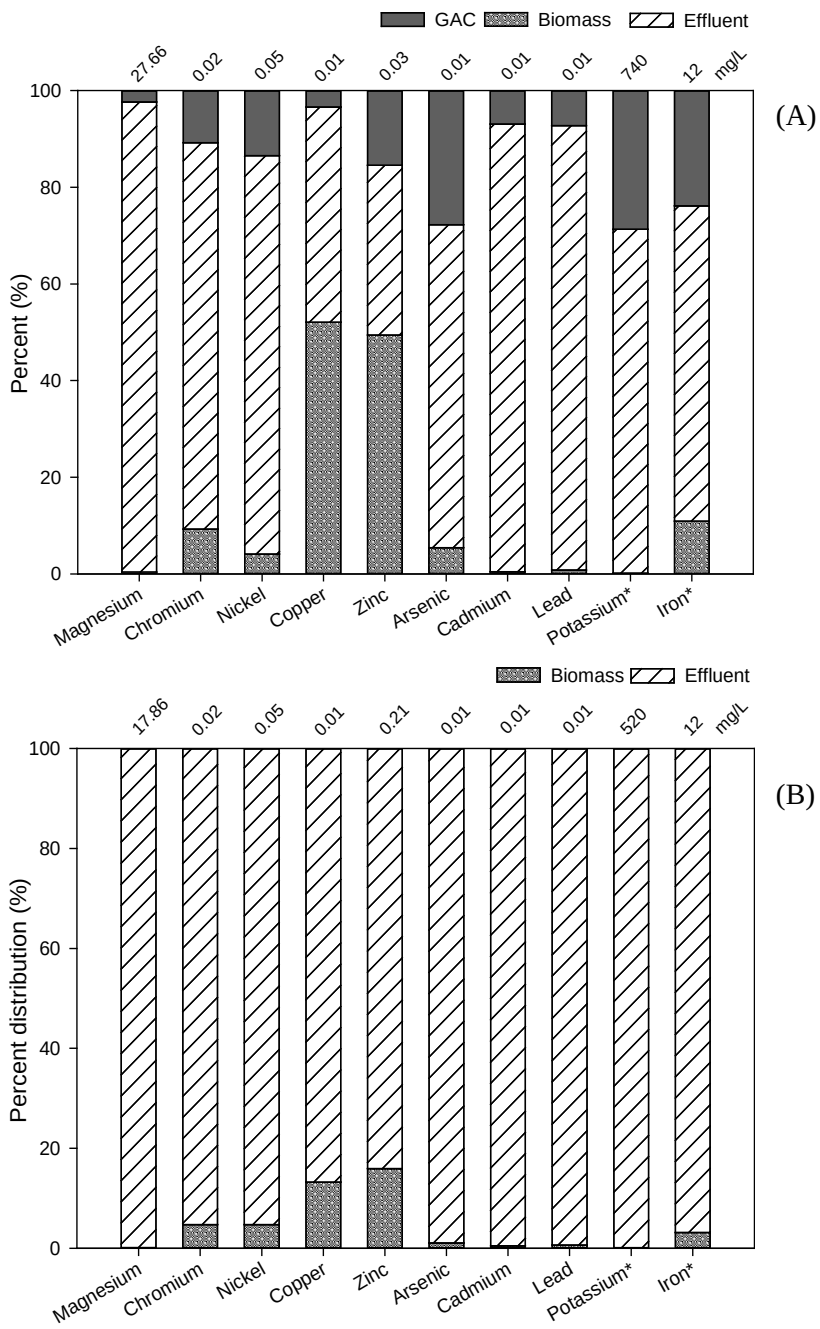


Figure 4.9 (A) Distribution of heavy metals in MABB with GAC and (B) in MABB without GAC.

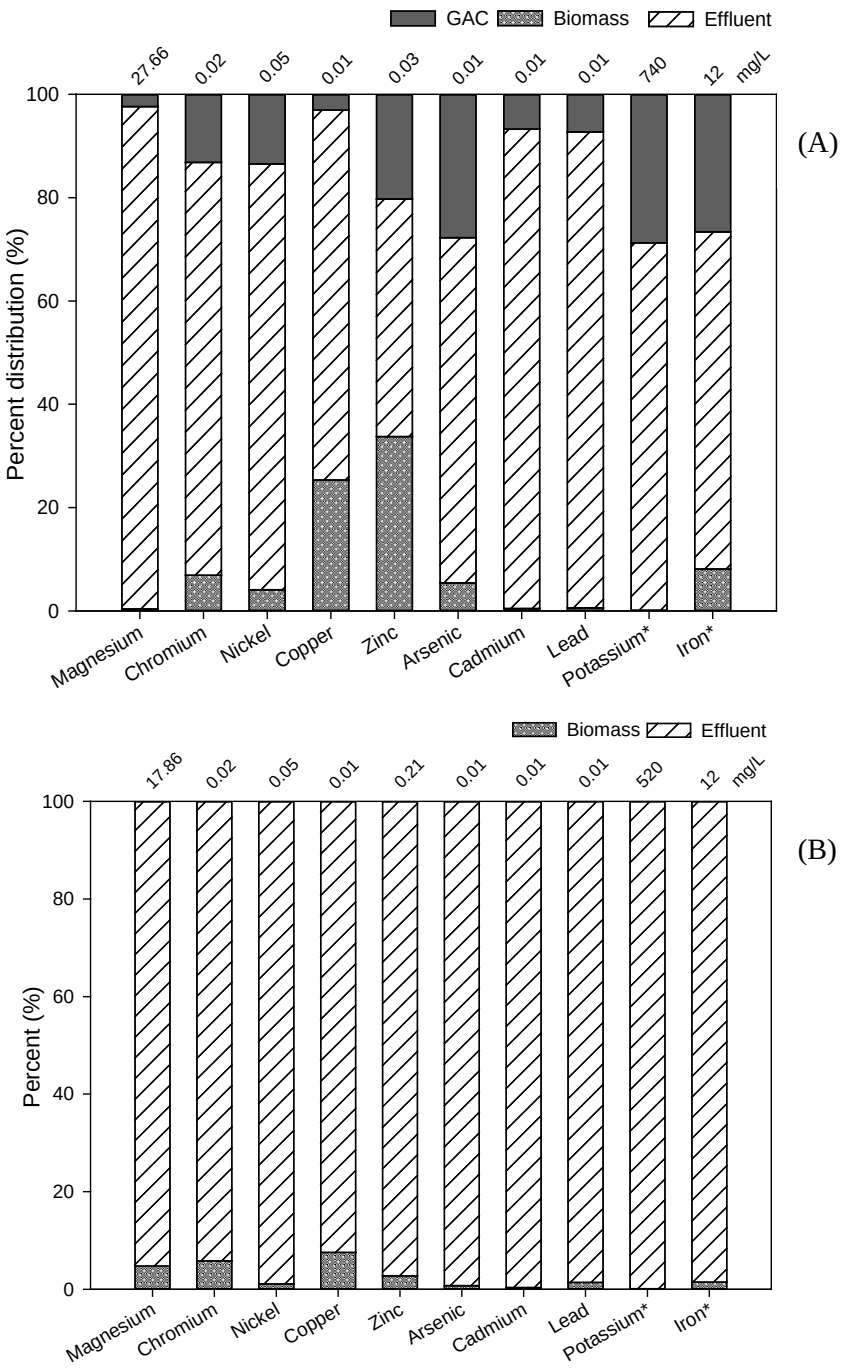


Figure 4.10 (A) Distribution of heavy metals in CAS with GAC and (B) in CAS without GAC.

4.3.3.2 CHO CELL MAMMALIAN CYTOTOXICITY

The CHO cell mammalian cytotoxicity analyses were conducted to quantify the wastewater cytotoxicity before and after MABB and CAS biological treatment both with and without GAC addition. According to Pham, (2013), there was a positive relationship between cytotoxicity and sCOD concentration in HTL-WW. Therefore, the sCOD of each sample was used to compare the relative cytotoxicity among the samples by calculating the organic concentration range per microplate well as µg/well. As shown in Figure 4.11, LPAM was cytotoxic compared to the concurrent negative control. Without spiking the biological reactors with CECs, the cytotoxicity in the effluents from MABB without GAC was decreased when compared to the LPAM influent, which indicated toxic compounds in LPAM were removed in the MABB. Thus, the cytotoxicity in the effluent from MABB and CAS with and without GAC were decreased after feeding bioreactors with LPAM. These results showed that the addition of GAC into bioreactors decreased cytotoxicity in the effluent from each MABB and CAS bioreactor, and the mean CTI value of samples were as follows:

MABB w/ GAC (17.4) < CAS w/ GAC (22.4) < MABB w/o GAC (41.8) < CAS w/o GAC (122.1)

When the GAC is added to the MABB and CAS bioreactors, it can reduce the cytotoxicity of the effluent by adsorbing toxic compounds. Figure 4.11 (B) also shows that MABB was more effective in reducing the cytotoxicity of LPAM than CAS. We suspect that the MABB reactor has an enhanced ability to uptake or degrade toxic compounds, but the exact mechanism of reducing cytotoxicity is not clear at this time.

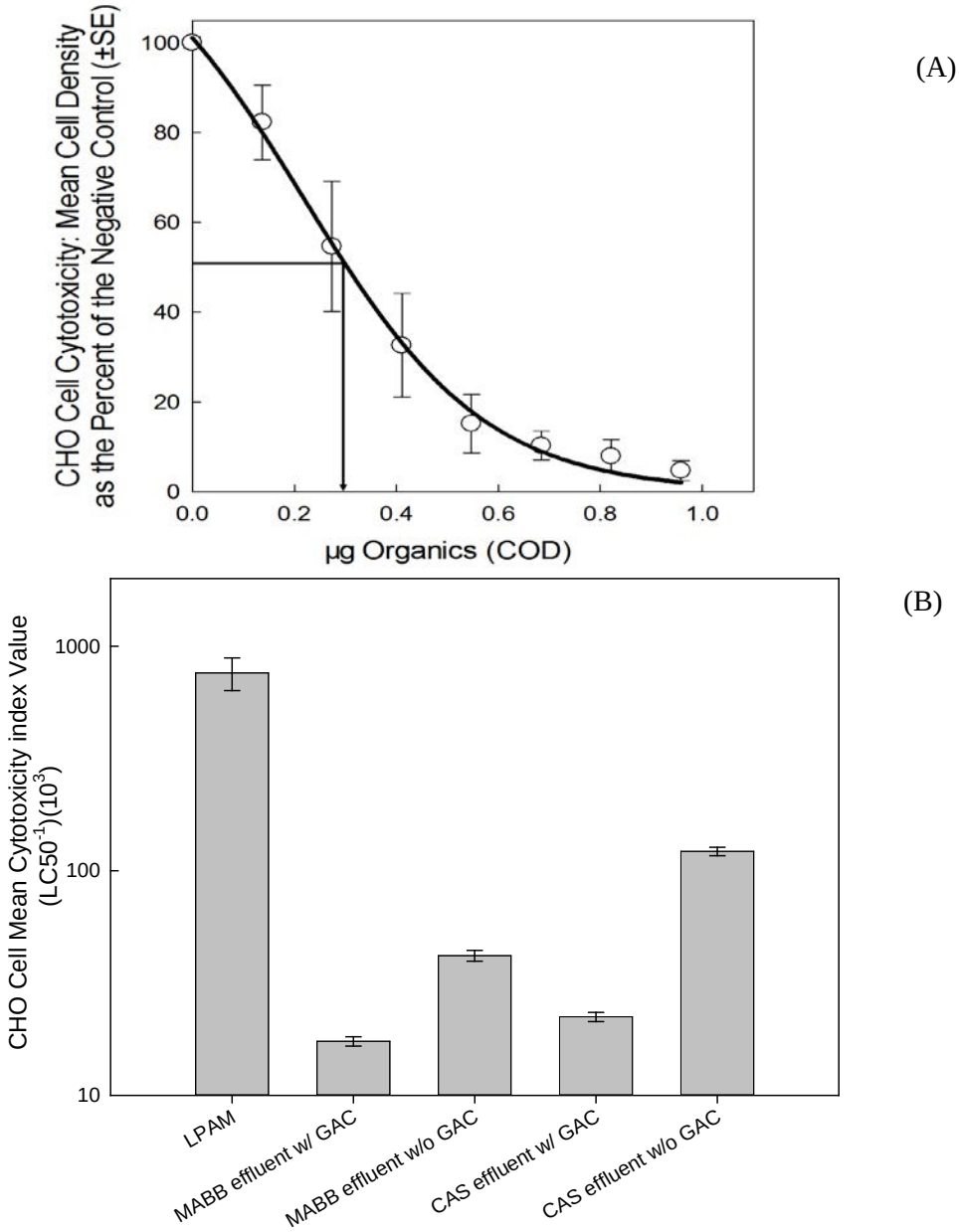
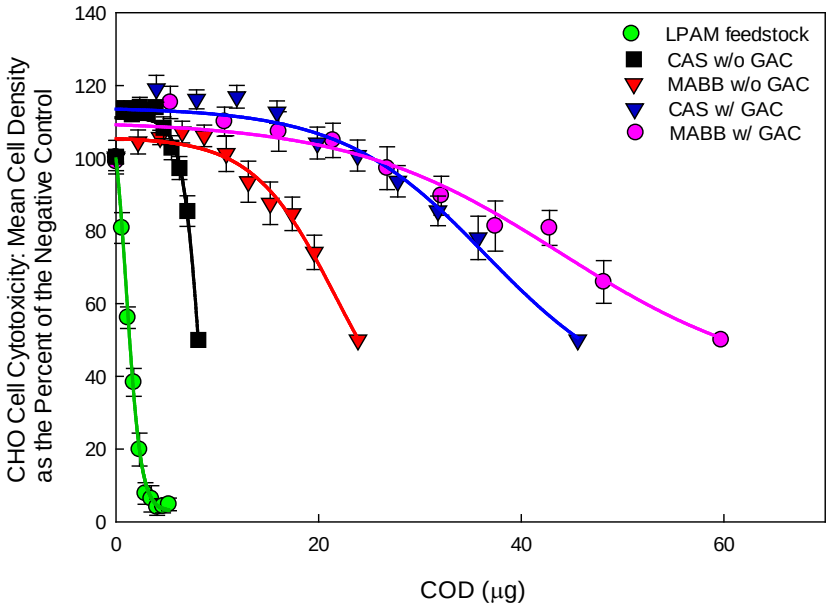


Figure 4.11 (A) Cytotoxicity concentration-response curve for CHG-WW (350°C/60/Ru) illustrating the regression of the data. The response at each concentration was generated from independent clones of CHO cells. The determination of LC_{50} value is indicated by the arrowed line (B) CHO cell Cytotoxicity Index Values (CTI) of inputs and outputs of MABB and CAS. The error bars indicate the standard error of the mean



(C)

Figure 4.11 (contd.) (C) A comparison of cytotoxicity concentration response curves from effluents of MABB and CAS. The error bars indicate the standard error of the mean

4.3.3.3 ACUTE TOXICITY

To quantify acute toxicity after MABB and CAS with and without GAC addition, a Microtox® assay was conducted. Compared to the effluents from the bioreactors, the LPAM feedstock was very toxic as shown in Figure 4.12. When compared to the original LPAM feedstock, the acute toxicity in the effluent was lower with and without GAC addition to MABB and CAS, which proves that toxic compounds in LPAM can be removed during biological processes. Thus, bioreactors with the addition of GAC demonstrated lower levels of acute toxicity in the effluent than each MABB and CAS bioreactor without the addition of GAC, and the normalized acute toxicity index value (MTI) were as follows:

MABB with GAC (Less than LOD) < CAS with GAC (22.1) < MABB without GAC (47.9) < CAS without GAC (70.2) < bLPAM (175.6)

These results draw two conclusions: First, toxic compounds for acute toxicity can be removed through biological processes such as MABB and CAS, but MABB was more efficient in removing toxic compounds in LPAM than CAS because of light deformation, higher temperature, and better removal of dissolved organics for biomass production. Second, GAC adsorption of toxic compounds was an effective way to decrease acute toxicity from LPAM feedstock. When the acute toxicity in bioreactors was analyzed with and without GAC addition, the statistical difference was significant (P=0.01) which can support the effectiveness of GAC addition for the removal of toxic compounds.

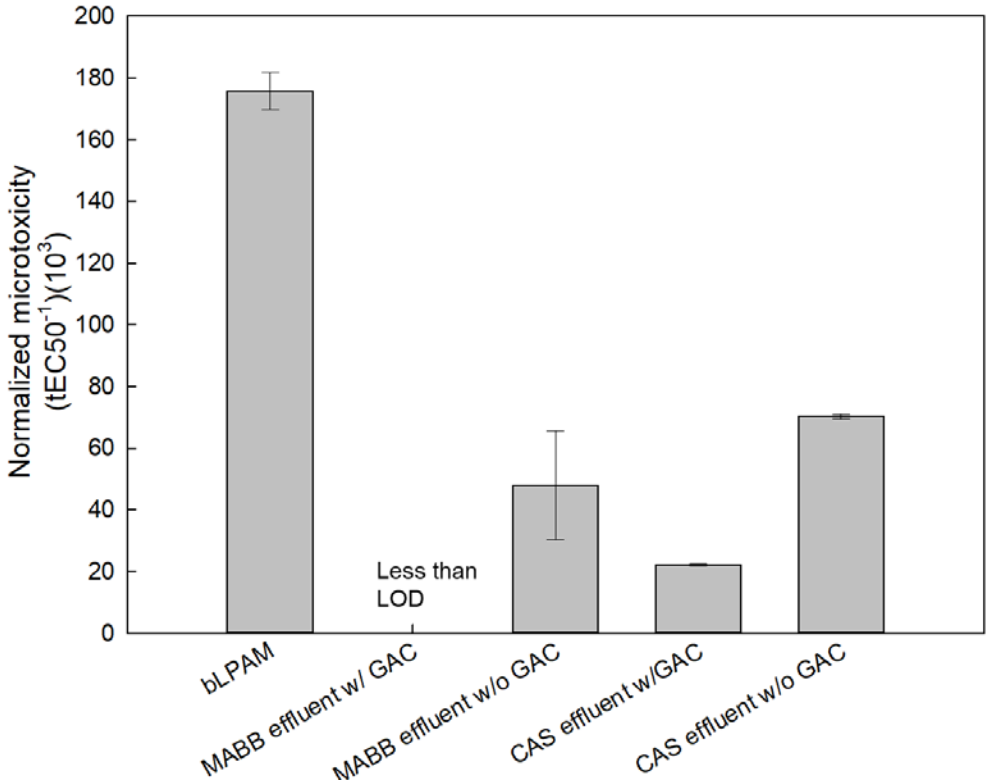


Figure 4.12 Normalized acute toxicity of inputs and outputs of bioreactors. The error bars indicate the standard error of the mean

4.3.3.4 YES YEAST CELL ASSAY FOR ESTROGENIC ACTIVITY

Using the Xenoscreen YES assay, the effluents from bioreactors, MABB and CAS, were investigated for estrogenic activity in the current study. An E2 concentration between 3.6 µg/L to 180 µg/L was the range of CECs used to spike the bioreactors for all the test. The yeast to the effluent from the bioreactors with and without GAC which showed agonistic response was displayed in Figure 4.13. The most potent among the four samples was CAS without GAC, followed by MABB without GAC according to their corresponding EC_{50} values. The estrogenic activity of LPAM influent could be reduced by GAC addition in the bioreactors, which could be similar as the removal of estrogenic hormones by GAC adsorption. By removing hormone compounds, GAC reportedly contributed to minimize estrogenic activity as supported by the results from Routledge and Sumpter (1996). Figure 4.13 (C) reported the EEQ of four different effluents ranging from 24.1 to 200.2 µg/L, which demonstrated an opposite trend of EC_{50}. Figure 4.13 (B) and (C) stated that the most potent sample, CAS without GAC, with the lowest EC_{50} and the highest EEQ. A similar trend was seen in the other samples as well.

Therefore, during biological processes, an effective way to remove hormone compounds and estrogenic activities from LPAM was through the addition of GAC. Additionally, MABB was a more effective reducer of estrogenic activity than CAS because its EC_{50} was higher than that of CAS.

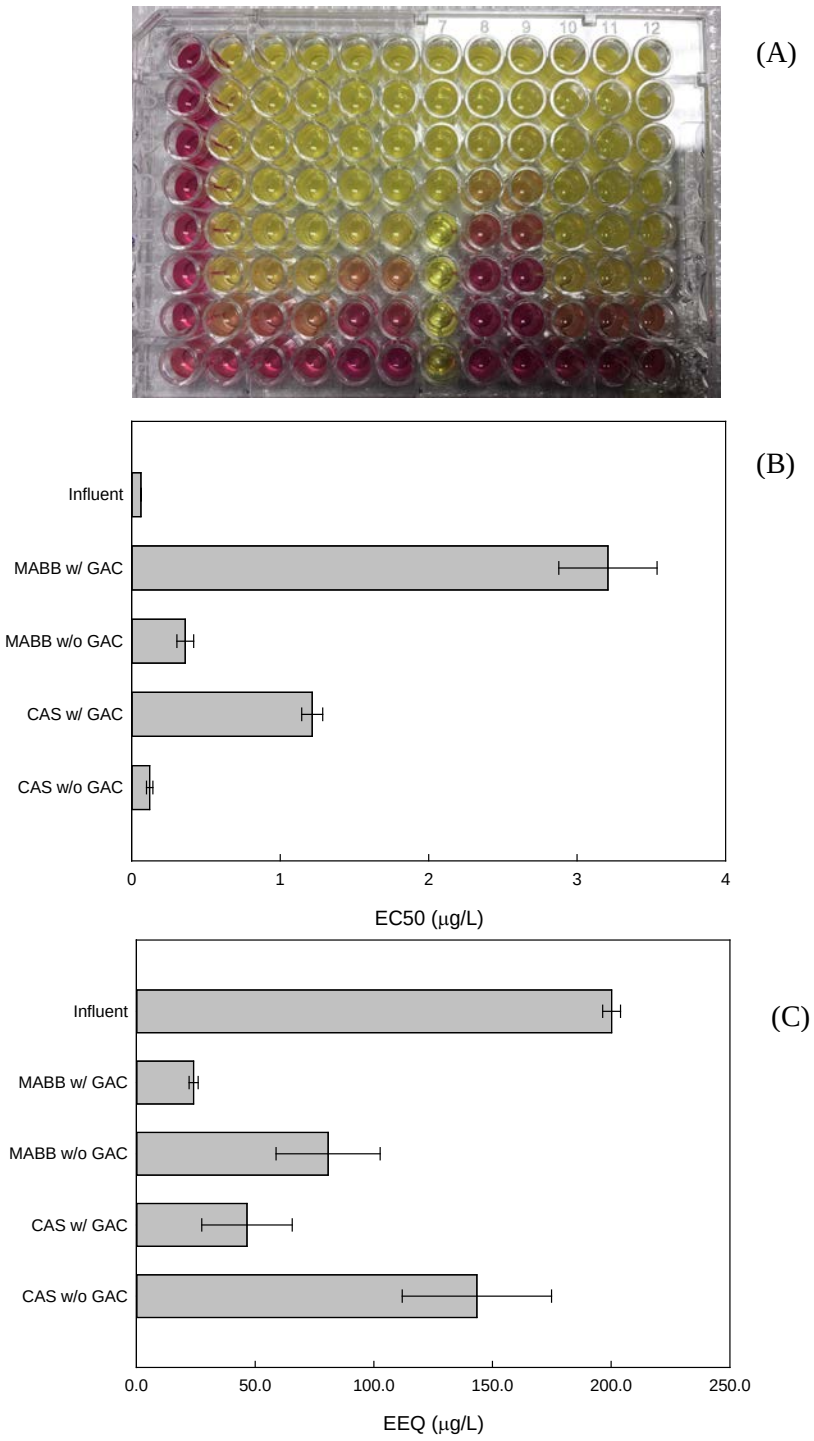
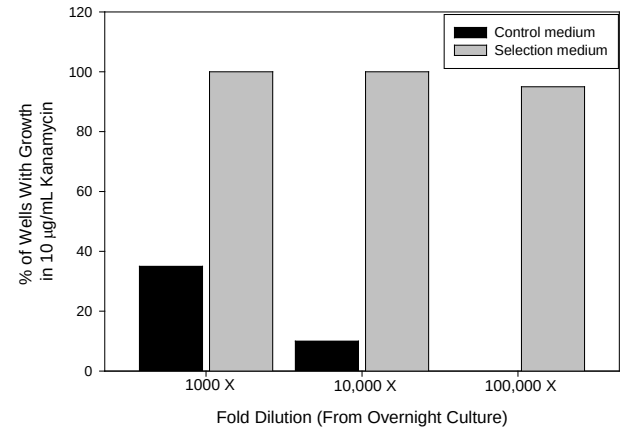


Figure 4.13 (A) Plate showing the response of the yeast screen to environmental estrogens in samples (B) EC_{50} for agonistic activities. (C) EEQ of the effluents from MABB and CAS with or without GAC addition. The error bars indicate the standard error of the mean

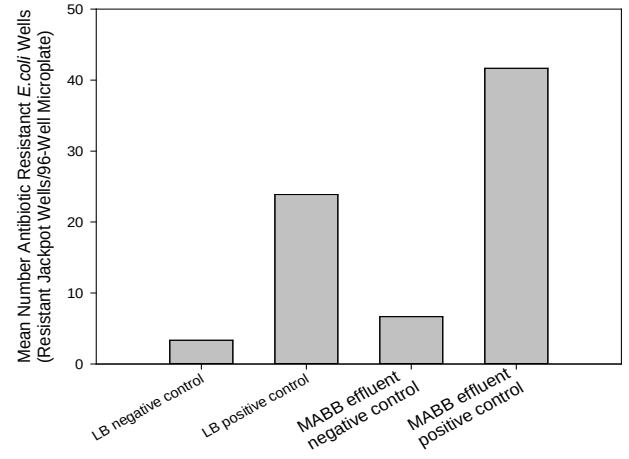
4.3.3.5 ANTIBIOTIC RESISTANCE ASSAY

A fluctuation test was developed to measure the antibiotic resistance in bacterial populations exposed to kanamycin and FF in manure. Figure 4.14 (A) shows that exposure to kanamycin at a non-lethal concentration increased survival during an antibiotic challenge (10 µg/mL). The results of this experiment showed that the bacteria grown in 3 consecutive overnight cultures of LB + 2 µg/mL kanamycin were more likely to produce growth in a lethal (10 µg/mL) concentration of kanamycin. This test was applied to LPAM for definition of baseline antibiotic resistance in a lethal (17 µg/mL) concentration of FF to develop a new analytical method was developed for the antibiotic resistance by FF. As shown in Figure 4.14 (B), exposure of *E.coli* to FF at a non-lethal concentration (2 mg/L) contributed to the increase in resistance to a lethal concentration of FF (17 mg/L), which is the antibiotic resistance to FF under LPAM+LB medium. There was a significant increase in the antibiotic-resistant bacteria jackpot wells of the E. coli that were grown under non-lethal FF selection (2 µg/mL) as an acclimation of E. coli to FF. The antibiotic resistance assay was sensitive and could detect selective pressures induced by concentrations as low as 2 mg/L FF. This study confirmed that exposure to FF at a non-lethal concentration increased survival during an antibiotic challenge.

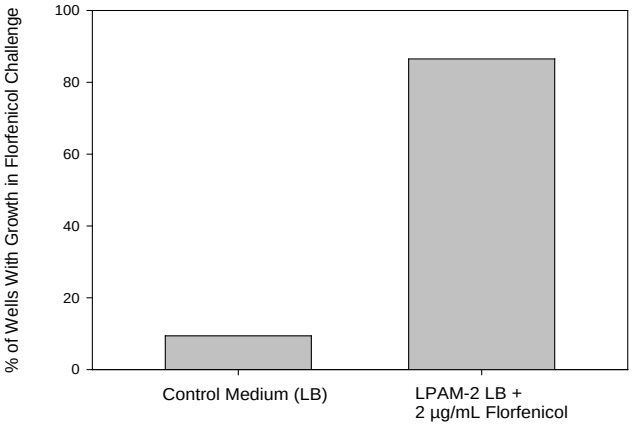
Thus, when the negative and positive controls of the LB medium and MABB effluent were compared in Figure 4.14 (C), the strong resistance was observed in the negative control in the LB medium and MABB effluent. These results described that three different types of medium, such as LB, LPAM, and MABB effluent, having different background matrices, could detect the antibiotic resistance response generated by a non-lethal concentration of FF.



(A)



(B)



(C)

Figure 4.14 (A) Data represent growth of E. coli grown for 3 overnight cycles in control medium (LB only) or selective medium (LB + 2 µg/mL) kanamycin plated into LB + 10 µg/mL. Growth in the selective medium conferred antibiotic resistance to the E. coli populations (B) Antibiotic resistance assay under LB medium and MABB effluent with positive and negative control (C) Culturing *E.coli* in medium containing 2 µg/mL FF increased resistance to a FF challenge (17 µg/mL)

4.4 CONCLUSIONS

When MABB and CAS were operated with and without GAC addition, sCOD removal ranged from 65% to 75% with similar performance between two reactors. However, after removing the GAC from each bioreactor, the removal of sCOD in the MABB and CAS were decreased by 11% and 12% in each reactor. The GAC was effective in removing recalcitrant compounds in the wastewater treatment process, which improved the organic concentration and biomass productivity. These results could be supported by reduced cytotoxicity due to GAC addition because sCOD was an index to determine the cytotoxicity in the effluents from bioreactors.

MABB showed better performance to remove phosphorus from LPAM than CAS with and without GAC. The addition of GAC into bioreactors enhanced the removal of total phosphorus, which supported the notion that the chemical adsorption of phosphorus onto activated carbon could be occurred and contributed to increasing the percent removal of total phosphorus in each bioreactor.

Removal of NH_3-N in the MABB and CAS were ranged from 92.8% to 97.4%, and adding GAC enhanced the removal up to 3.7% and 2.3% in MABB and CAS, respectively, which described that all the effluents only with GAC met the discharge standard of NH_3-N, 2.5 mg/L (IPAC, 2014). Furthermore, percent removal of TDN in the MABB and CAS with GAC addition were increased up to 9.9% and 9.4% in MABB and CAS, thus, reduction of TDN was mostly caused by biological assimilation (Liao et al., 1993; Nelson et al., 2003).

After removing GAC, the biomass productivity of MABB and CAS were decreased by 23% and 8.7% in MABB and CAS, which meant that GAC could improve the biomass productivity by removing recalcitrant and inhibiting compounds. Furthermore, the ash content was decreased with GAC addition, which means that GAC could enhance bioenergy yield in hydrothermal processes because ash is not useful for bioenergy production (Biller et al., 2012).

Percent removal of total hormones in the bioreactors were ranged from 81% to 97% on average, and MABB showed better performance to remove hormones than CAS because the higher temperature and LED light in MABB could contribute to enhance the hormones removal by the light deformation of hormones. In addition, GAC increased the removal of total hormones up to 10.9% and 8.1 % in MABB and CAS, respectively, due to additional adsorption of hormones to GAC (Westerhoff et al., 2005; Yoon et al., 2003). These results stated that GAC addition could enhance the removal of estrogenic hormones in the bioreactors, and MABB was more synergistic with GAC to remove hormones than CAS due to higher temperature. Adding GAC to biological processes contributed to enhance the removal of estrogenic activities as well as hormone compounds in the bioreactors. Thus, the EC_{50} of MABB was higher than CAS, which meant MABB was more effective to reduce estrogenic activity than CAS.

More than 91% of FF could be removed in the bioreactors, and MABB showed higher FF removal on average than CAS especially without GAC addition, but the difference in the percent removal was not significant (P=0.12). Thus, the removal of 4-MSB and 4-MSAP were sensitive to the addition of GAC than MPS because the distribution of 4-MSB and 4-MSAP to effluents from the bioreactors were increased from 2.1% to 5.6% and from 10.4% to 27% with GAC addition.

Heavy metals in the inputs and outputs of MABB before and after GAC addition were analyzed, and GAC enhanced the percent removal of heavy metals as much as 5.6% from LPAM. Thus, heavy metals in the MABB effluents contained 94% to 98% of total heavy metals, but the concentrations were significantly lower than the recommended standard of Cu and Zn for irrigation or water reuse (Ayers et al., 1985).

MABB showed higher removal of acute toxicity and cytotoxicity than CAS due to light deformation, higher temperature, and better removal of dissolved organics for biomass production.

Thus, GAC addition contributed to enhance the effluent water quality by removing toxic compounds because of extra adsorption capacity, which resulted in the decreased acute toxicity and CHO cell cytotoxicity.

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CHAPTER 5. EFFECTS OF OPERATING CONDITIONS OF HYDROTHERMAL BIOFUEL PROCESSSES ON THE FATE OF BIOACTIVE CECS

5.1 INTRODUCTION

The extensive expansion of CAFOs over the past few decades have created environmental concerns related to the increase in the quantity of animal manure in concentrated areas (Ro et al., 2007). Animal manure contains a variety of chemical compounds which, if released into the environment without treatment, can negatively impact the environment. For instance, animal waste can also contain a concentrated dose of various estrogens that can be introduced into the food chain by plant uptake or seep into natural water sources after land application of manure (Subbiah et al., 2011). These estrogenic compounds can have adverse effects on the reproductive biology of aquatic vertebrates (Irwin et al., 2001; Routledge et al., 1998; Schuh et al., 2011; Subbiah et al., 2011). A process to deactivate or destroy residual estrogenic compounds in animal manure is investigated in this study to ameliorate the potential for environmental pollution.

HTL is a novel waste-to-energy system that can be integrated with algal biomass production during wastewater treatment. HTL converts wet biomass of algal and bacterial biomass into bio-crude oil, which is a biofuel intermediate that is akin to crude petroleum. Also, the HTL process can effectively convert bioactive organic compounds into bioenergy products or otherwise break them down into inactive forms (Pham. 2013). According to Gai et al., (2015), the key and independent variables in HTL process are temperature, retention time, and solids ratio that can affect HTL performances including nutrient recovery. In this study, temperature and retention time were selected as main parameters to investigate the removal of CECs, and the levels of two

operating parameters were determined within the ranges of 200-400°C and 30-60 minutes, respectively, based on the results of preliminary experiments (Yu. 2012).

Another possible method to treat animal manure is the use of catalytic hydrothermal gasification. This is a thermo-chemical conversion method that could simultaneously deactivate estrogenic compounds and produce useable biogas, which could reduce dependency on fossil fuels. Residual organics treatment with gasification technology is supported by the Gas Research Institute (GRI) and U.S. Department of Energy (DOE) (Ro et al., 2007). This technology is capable of treating organic-laden wastewaters and wet biomass under catalytic hydrothermal processing conditions (250 - 360°C, up to 22 Mpa) (Elliott et al., 1988; Elliott et al., 1994). It converts the majority of organic contaminants to gaseous compounds and minimal quantities of char (Balat et al., 2009; Ro et al., 2007). The gaseous products, called syngas, is a mixture of carbon monoxide (CO), carbon dioxide (CO_2), hydrogen (H_2), and methane (CH_4). Additionally, hydrothermal gasification uniquely offers the advantage of processing wet manure feedstocks, minimizing the need for pretreatments, which improves the cost-effectiveness of treating large volumes of manure (Ro et al., 2007).

Various heterogeneous catalysts are effectively used in the catalytic gasification of organic compounds (Azadi & Farnood. 2011). Catalytic hydrothermal gasification is effective over a wide range of temperatures, pressures, and input catalysts. In previous studies done on the catalytic gasification of biomass, experiments with a Ruthenium catalyst produced more than twice the gas yield of the control (Onwudili & Williams. 2013). Highly efficient carbon gasification systems can be constructed using the ruthenium catalyst in supercritical water gasification at 400°C (Brown et al., 2010). In a previous research, iron, cobalt, ruthenium, and nickel catalysts were used in the gasification of biomass. Catalysts not only improve the quality of gas products and the efficiency

of conversion, but they also reduce tar content (Balat et al., 2009). Another study on the gasification of corn starch showed that the addition of a homogeneous potassium catalyst improved the efficiency of converting certain types of biomass to syn-gas, but had no effect when clover grass or corn silage was converted (D'Jesus et al., 2005). This demonstrates that different catalysts are optimal at producing biogas depending on the type of biomass. Also, a catalyst which optimizes gas production could also be useful in treating estrogenic compounds in animal manure. To discover the best approach for deactivating estrogenic compounds, the effects of HTL and CHG operating conditions on the fate of bioactive CECs were investigated. Therefore, this study will investigate the fate of CECs from liquid swine manure, which accounts for 80% - 95% of total manure quantities, which are subjected to hydrothermal bioenergy conversion processes (MWPS-18. 2004).

The specific objectives of this study were to 1) investigate the effects of operating parameters in HTL and CHG on the fate of estrogenic hormones and FF in the biomass, 2) identify the key operating parameters for each hydrothermal process affecting the removal of CECs and bioenergy production, 3) develop an analytical method to quantify the breakdown products of FF, and 4) evaluate the correlation between operating parameters and the overall estrogenic activity or antibiotic resistance capacity.

5.2 MATERIALS AND METHODS

5.2.1 CHEMICALS AND REAGENTS

Estrone (E1, ≥99%), 17β–estradiol (E2, ≥98%), estriol (E3, ≥97%), and 17α–estradiol (EE2, ≥98%) were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI). The florfenicol (FF, No. F1427) and florfenicol amine standard (FA, No. 32492) were procured from Sigma-

Aldrich and had a purity of 99.8% or greater. Stock solutions of E1, E2, E3, and FF at 2 mg/mL were prepared in pure methanol. SPE columns, *Supelclean Envi-Carb* (500mg/6mL, No. 57094) and *Supelclean* LC-Florisil (1g/6mL, No. 57057), were purchased from Supelco (Bellefonte, PA, USA) and used for extracting estrogenic hormones and removing background matrix. N-Butaneboronic acid (No. 16324-4) and N, N-Dimethylformamide (DMF, No.68-12-2) were purchased from Sigma-Aldrich and VWR with a purity of 97% and 99.7%, respectively, for the derivatization of FF in the dried samples.

The FF breakdown products such as 4'-(methylsulfonyl) acetophenone (4-MASP, No. 549304), 4-(methylsulfonlyl) benzaldehyde (4-MSB, No. CDS003885), and methyl phenyl sulfone (MPS, No. 68742) were purchased from Sigma-Aldrich and had a purity of 99.8% or greater. Distilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade organic solvents such as n-hexane, acetone, acetonitrile, methanol, or dichloromethane were purchased from Fisher Scientific (Fair Lawn, NJ). All Chemicals and solvents in this research were purchased at a HPLC grade. Two metal catalysts, Ruthenium on alumina and Raney Nickel, were purchased from Fisher Scientific (Hanover Park, IL, No. AA1174922) and Sigma–Aldrich (Milwaukee, Wi, No.221678). NaOH was purchased from Sigma – Aldrich (Milwaukee, Wi, No.221465) and made to 2 mol/L.

Figure 5.1 Tubular reactor and gas meter for HTL and CHG tests

5.2.2 FEEDSTOCK FOR HYDROTHERMAL PROCESSES

To study the fate of hormones during HTL and CHG processes, artificially spiked biomass (E1, E2, and E3, 3 mg/L for each compounds) was used in this study. Wet mixed biomass was collected using a flat-sheet Kubota microfiltration membrane (0.4 μm, cartridge type 203) from MABB and stored at 4°C before processing. The solids content and volatile solids percentage of the homogenized biomass after a high shear mixer was 19.3±0.2 % and 81.9±0.04%, respectively. Before HTL and CHG of biomass, the concentrations of E1, E2, E3 and EE2 in the biomass and HTL-WW were analyzed using the analytical method for solid and liquid fraction. A detailed method for biomass preparation and hormones analysis was explained in Chapter 3.

5.2.3 HYDROTHERMAL LIQUEFACTION

To define the optimal condition for bioenergy production and the effects of HTL on the fate of estrogenic hormones in the biomass, four different reaction temperatures (250, 300, 350, and 400 °C) all with the same reaction time of 60 minutes were applied in HTL tests with hormone spiked biomass. The major products of hydrothermal biomass conversion included biocrude oil, solid residues, and aqueous products which was used to analyze the residual hormone concentration after the reaction. All the HTL treatments were investigated using a small tubular batch reactor assembled with parts from Swagelok. The reactor was made of a 3/4 inch OD tube (0.095-inch wall thickness) sealed with a cap and a needle valve with necessary fittings. Tubing was cut to length for 40 mL of working volume; approximately 5 mL of extra dead volume was attributed to fittings and valve. 10g (total weight) of biomass was loaded in the home-made Swagelok tubular reactor, then the reactor was purged with nitrogen three times, and finally sealed at 90 psi. Afterwards, the reactor was placed inside a preheated furnace (Barnstead Thermolyme Co.) at designated temperature. After the desired reaction time elapsed, the reactor was submerged into a bucket of water at room temperature to cool down for at least 3 minutes. Then the reactor was opened, and the contents were carefully collected after it reached room temperature.

Biocrude oil yield, dry wt. %

$$Y_O = \frac{m_O}{m_F(1-W)} \times 100 \quad (5\text{-}1)$$

Solid product yield, dry wt. %

$$Y_S = \frac{m_S}{m_F(1-W)} \times 100 \quad (5\text{-}2)$$

Y_o: yield of biocrude oil

Y_s: yield of biocrude solid

m_o: mass of biocrude oil

m_s: mass of biocrude solid

m_F: mass of biocrude feedstock

W: water content of the feedstock

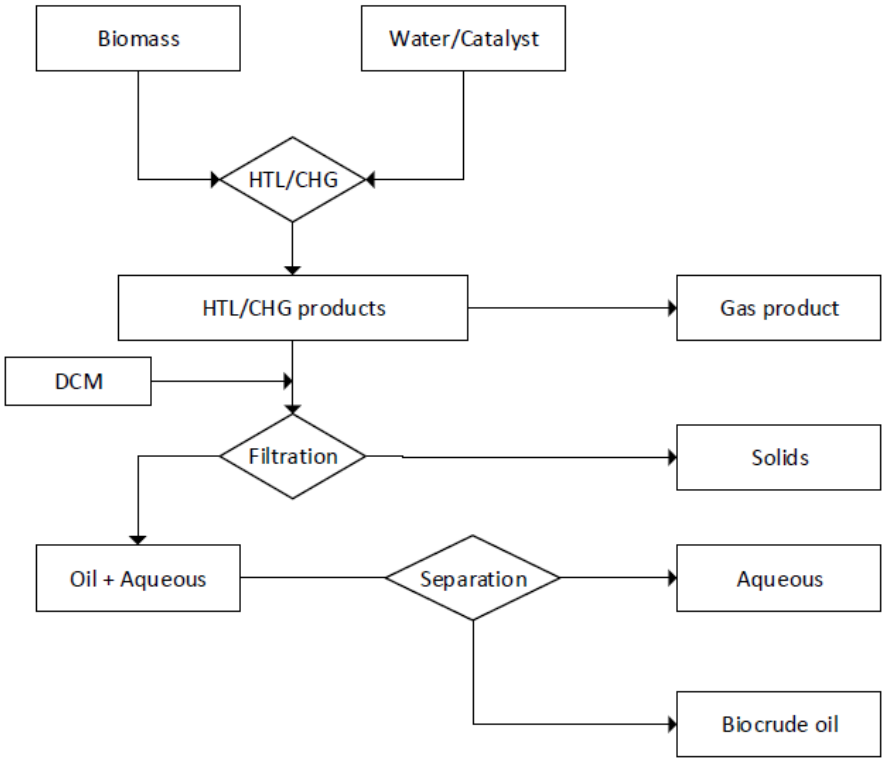


Figure 5.2 Product separation for hydrothermal liquefaction (HTL) and catalytic hydrothermal gasification (CHG)

As shown in Figure 5.2, the solid, aqueous, and oil products were mixed with 10 mL dichloromethane (DCM) in the reactor body which was then placed on a shaker at 1000 rpm for 10 seconds after the HTL reaction. The contents were transferred to a vacuum filter (Whatman No. 4 Filter Paper) for separation. The reactor body was further rinsed with DCM in 10 mL portions and shaken until the decanted liquid was clear. After filtration, the solids remained on the filter while the aqueous and oil (in DCM) fractions were collected in the flask. Next, the aqueous and oil (in DCM) fractions were separated by a separatory funnel, and they were transferred to sealed vials. The yields of biocrude oil and solid residue were calculated by equation 5-1 and 5-2, neglecting the small production of gas.

Table 5.1 Operating conditions for hydrothermal liquefaction tests

		Hydrothermal Liquefaction (HTL)			
Temperature (˚C)		**250**	**300**	**350**	**400**
Reaction time (minute)	**30**	250/30	300/30	350/30	N/A
	60	250/60	300/60	350/60	400/60

To check the organics removal and break down of hormones during HTL tests, four different reaction temperatures (250, 300, 350, and 400 °C) with reaction times of 60 minutes were applied to HTL tests with spiked biomass. Reaction times of 30 minutes with four different temperatures of HTL tests were investigated to analyze the yield of biocrude oil. Table 5.1 displays the combinations of HTL operating conditions to test the effects of HTL on the fate of estrogenic hormones in the mixed biomass and the yield of biocrude oil.

5.2.4 CATALYTIC HYDROTHERMAL GASIFICATION

To investigate the effects of CHG operating conditions on the fate estrogenic hormones, CHG experiments of biomass were conducted under 16 different combinations of operating conditions including reaction temperature (350 - 600°C), reaction time (15/30/60/90 minutes), catalyst type (Ru / Ra-Ni / NaOH / Ru+NaOH / Ra-Ni+NaOH), and catalyst amount (0.1, 0.5, 1, 2, 4g catalyst/10g biomass). Direct biomass gasification tests were conducted using the same 40 mL Swagelok batch reactor as HTL tests. MABB biomass was premixed with ruthenium on alumina (Ru) catalyst (10% of biomass weight) before being added into the reactor. The reactor was loaded with the 10g of homogenized biomass and 0.5 mL of the stock solution (E1, E2, and E3: 0.5 mg/mL; FF 1 mg/mL). DI water without stock solution served as the blank control. After loading the spiked feedstock, the reactor was purged 3 times with pure nitrogen and pressurized at 90 psi to keep the consistency with HTL runs. The furnace (Barnstead Thermolyme Co.) was preheated and maintained at the designated temperature for each reaction. Finally, the reactor was placed into the preheated furnace for the desired reaction temperature. Four reactors were simultaneously run under various operating conditions of the same temperature as shown in Table 5.2, and the destruction of estrogenic hormones were recorded for each variation.

Afterwards, a water bath at room temperature was prepared to quickly cool down the reactors. The gas volume was measured by reading the pressure from the attached gauge, and 10 mL of the gas was kept in a vacuum gas sample vial for further analysis. HTL-WW samples from each reactor were collected and placed into glass vials for GC/MS analysis followed by SPE. All the samples collected from each operating condition of CHG were prepared for hormone analysis within 2 hours after being deposited according to the analytical methods in Chapter 3 to obtain

accurate data of hormones before occurring the transformation. In addition to biocrude oil and solid residue yield, the overall gas yield was calculated based on equation 5-9.

Table 5.2 Operating conditions for catalytic hydrothermal gasification tests of biomass and HTL-WW

Operating parameters		**Catalytic Hydrothermal Gasification (CHG)**					
Temperature (˚C)		350	400	450	500	550	600
Reaction time (minute)	**15/30/60/90**	350/15/30/60/90	-	-	-	-	-
	60 (general)	350/60	400/60	450/60	500/60	550/60	600/60
Catalyst type		Ru / Ra-Ni / NaOH / Ru + NaOH / Ra-Ni + NaOH					
Catalyst amount (g catalyst/10g biomass)		0.1 / 0.5 / 1 / 2 / 4					
Feedstocks		Biomass (MABB/CAS) or HTL-WW(MABB/300˚C/60minutes)					

Direct catalytic hydrothermal gasification of cultivated biomass was conducted at three selected conditions (400°C, 500°C, and 600°C at 60 minutes) to investigate the gas production and energy recovery. Homogeneous catalyst (2 mol/L sodium hydroxide), and heterogeneous catalysts (Ra-Ni and Ru) were used for the tests at loading amount of 2 mL and 0.5 g, respectively, with 10 g total wet biomass. Hydrogen, methane, carbon dioxide, and carbon monoxide were the main components in the gas products of the direct CHG of the cultivated biomass, with hydrogen and methane being the primary energetic gases. Carbon monoxide was not detected when temperatures were below 400°C. For each CHG test, the ideal gas law at room temperature determines the total

gas volume according to reactor volume and final gas pressure measurement after performing the CHG reaction. GC/MS determined the gas composition (H_2, CH_4, CO_2, and CO) as a percentage by volume, and thus, each produced gas volume could be calculated. Equation 5-3 calculated the overall gas yield.

Gas yield from biomass, dry wt. % $$Y_G = \frac{\sum(V_{tot} \times V_i\% \times M)}{22.4 \times m_F(1-W)} \times 100 \qquad (5\text{-}3)$$

In which Y_G represents the gas product yield from CHG tests performed on the biomass. m_F and W represent the mass and water content of feedstock, respectively. he total gas volume and the volume percentage for each gas *i* is represented by V_{tot} and V_i%, respectively. The molar mass of the gas mixture is represented by M, which, under average gas composition of CHG gas products, is estimated at 25 g/mol.

A CHN analyzer (Exeter Analytical, Model CE440) characterizes the contents (wt. %) of the carbon, hydrogen, and nitrogen. Assuming a negligible quantity of other elements present in the same for this analysis, the oxygen content was determined based on the difference. The HHVs (Higher heating value) of the biogas/biocrude oil product calculated the energy balance. Channiwala and Parikh's formula determined the HHVs of the feedstock and biocrude oil (Channiwala and Parikh, 2002), depicted below:

HHV = 0.3491C + 1.1783H + 0.1005S – 0.1034O – 0.0151N - 0.0211A (MJ/kg)

in which C, H, S, O, N, A represent carbon, hydrogen, sulfur, oxygen, nitrogen, and ash contents in the sample by dry weight mass percentages. The standard HHVs of each unique gas

multiplied by its corresponding volume yield determined the HHVs of each gas product. Using equation 5-4 and 5-5, the energy recovery (ER) of each energy product was calculated.

Energy recovery (oil), %	ER_O	$ER_O = \frac{HHV_O \times Y_O}{HHV_F} \times 100$	5-4
Energy recovery (gas), %	ER_G	$ER_G = \frac{\sum_i HHV_{G,i} \times Y_G}{HHV_F} \times 100$	5-5

where ER_o and ER_G represent biocrude oil and gas product energy recovery, respectively, from the feedstocks. The HHV of biocrude oil, feedstock, and each individual gas product *i* is represented by HHV_O, HHV_F, and $HHV_{G,I}$, respectively. The yield of biocrude oil and gas products is represented by Y_o and, Y_G, respectively. All the analysis about the biomass components and bioenergy production was performed by Peng Zhang.

5.2.4.1 CHG OF BIOMASS WITH DIFFERENT TEMPERATURE AND REACTION TIME

According to preliminary HTL experiments with biomass, biocrude oil yield reached the highest value of 39% (dry basis) at 300˚C/60minutes. With higher temperatures in biomass CHG, the gasification process became intensified and biocrude oil was partially converted in to gaseous products such as CO, CO_2, H_2, and CH_4, thus the oil yield was lower. The increased temperature would affect the removal of bioactive CECs, and the percent removal of each hormone and FF will be analyzed to investigate the correlation between gasification with higher temperature and the fate of bioactive CECs.

According to Susanti et al., (2011), longer reaction time could increase the hydrogen yield, but methanation can be occurred with further reaction time. Also, longer reaction time can contribute to produce higher gas yield, which means more complete gasification of biomass, and

reaction time had no significant effect on hydrogen yield when compared to the effects of temperature and pressure since the CHG reaction was very rapid in the range of experimental operating parameters (Lu et al., 2012). Based on the previous studies, the longer reaction time could enhance the conversion of biomass to gas, which means the higher removal of bioactive contaminants from the biomass feedstock. Therefore, the removal of CECs under increasing temperatures and reaction time is expected to be accelerated and enhanced with significant relationship ($P<0.05$).

5.2.4.2 CHG OF BIOMASS WITH DIFFERENT TYPES AND AMOUNT OF CATALYST

To investigate the effects of catalyst on the fate of CECs under CHG, five different combinations of two metal catalysts (Ru and Ni) and one alkali catalyst (NaOH) were used for biomass CHG. NaOH as a base catalyst in this study offer better performance in biomass CHG, but it is difficult to recover them from the aqueous phase (Basu. 2013).

According to Yoshida and Oshima, (2004), sufficient amount of catalyst achieves high gasification efficiency for the mixtures of cellulose and softwood lignin, which gives better results for biomass gasification. However, some literature suggested that the reaction is zero-order over the amount of catalyst (Ru/C) in the reaction with model compounds as feedstock at a relatively lower organic loading level. Therefore, the destruction of bioactive contaminants could be proportional to the amount of catalyst because the more gasification of organics means the better breakdown of CECs. Five different amounts of Ru catalyst were used in these tests, and they were from 0.1 to 4g per 10g biomass to cover the wide range of catalyst and biomass ratio from 1:100 to 1:2.5.

5.2.5 SEQUENTIAL HTL AND CHG OF BIOMASS

To perform the preliminary tests of combined HTL and CHG operations to assess the performance of sequential treatment on the bioactive CECs removal in the biomass, the reaction temperature was increased from 350°C to 600°C for CHG of HTL-WW, which was produced from the biomass HTL under 300°C/60minutes. Thus, the estrogenic activity and antibiotic resistance of HTL/CHG-WW will be investigated to evaluate the validity and environmental impacts of water reuse.

5.2.6 ANALYSIS OF BIOACTIVE CECS IN HTL-WW AND CHG-WW

5.2.6.1 ESTROGENS AND ESTROGENIC ACTIVITY

The residual E1, E2, E3 and EE2 in the post-HTL and CHG wastewater were extracted and purified based on the SPE method for liquid samples. All HTL-WW and CHG-WW samples after the HTL and CHG of biomass were analyzed using GC/MS after derivatization as stated in the procedure in Chapter 3. Thus, activating (agonistic) activities of HTL-WW and CHG-WW were quantified in this study using the Xenoscreen YES cell assay to investigate the effects of various operating conditions of HTL and CHG on the fate of estrogens and estrogenic activity as stated in Chapter 4. A sign of agonistic activity was indicated by the induction of the response parallel to this fixed agonist concentration. The manufacturer protocol of the Xenoscreen XL assay outlines the methods of calculation for the induction. EEQ expressed the estrogenic potency of the compound or environmental extract. Also, EC_{50} values, the concentration when half of the highest estrogenic effect was detected, were used to express the estrogenic potency of a compound relative to E2. Finally, CECs removal efficiency and environmental safety of the integrated manure

management will be evaluated using the residual concentration of hormones and estrogenic activity assay.

5.2.6.2 FLORFENICOL AND ANTIBIOTIC RESISTANCE

The concentration of residual FF in the HTL-WW and CHG-WW after the hydrothermal bioenergy processes with various operating conditions. After the hydrolysis and extraction of FF in the wastewater samples, the FA was analyzed using GC/MS after derivatization in Chapter 4.

To examine the hydrothermal effects on the removal of antibiotic resistance, a fluctuation tests were executed to measure antibiotic resistance in HTL-WW and CHG-WW. The biomass feedstock was spiked with 1 mg/mL FF and applied to hydrothermal processes to produce aqueous phase from each HTL and CHG process. The positive and negative control of HTL-WW and CHG-WW were compared to quantify the antibiotic resistance generated by FF. All the analytical processes followed the method, which was previously developed by professor Plewa's research group.

5.2.6.3 DEVELOPMENT OF ANALYTICAL TECHNIQUE FOR FLORFENICOL BREAKDOWN PRODUCTS

HTL degradation products or changes in the FF to the right of the nitrogen portion were not identifiable. However, FA was still detectable in the outputs of the hydrothermal processes. DCM and final DMF were thus carefully examined, being the only sources of observable breakdown products of FF. These fractions were the focus for determination of key breakdown products of FF by the hydrothermal processes.

The DCM portions were concentrated to a final volume of 1 mL, scanned by low resolution GC/MS and the spectra collected from 100 m/z to 300 m/z. The GC inlet and separation parameters

were identical to those used for the high-resolution FA measurements in Chapter 4. The data obtained from the HTL feedstock extracts were subtracted from those of the HTL-WW extracts. A peak was observed in the post-processed DI water and swine manure samples at a retention time of 6.08 minutes that was not present in the HTL-feedstock extracts. According to the National of Institute of Standards and Technology (NIST) 2011 mass spectra database, it was probably 1-[4-(methylsulfonyl)phenyl]- ethanone (CAS# 10297-73-1). A high-resolution GC/MS experiment was performed for an accurate mass measurement at m/z 198.0351, and it was confirmed that the formula of the peak was $C_9H_{10}O_3S$.

General scan GC/MS analysis of the DCM samples indicated three breakdown products present in the HTL product samples. These products included 4'-(methylsulfonyl) acetophenone (4-MSAP, CAS#10297-73-1), methyl phenyl sulfone (MPS, CAS#3112-85-4), and 4-(methylsulfonyl) benzaldehyde (4-MSB, CAS#3446-89-7). To positively identify the peak at the retention time of each compound, the breakdown products were purchased from Sigma-Aldrich. A 100 mg/L FF in DI water was prepared and treated at two different HTL conditions (300°C/30minutes and 220°C /20minutes). After the HTL tests, 2 mL of the stock FF and FF HTL products were added to a TurboVap concentrator, and then the water was removed at 40°C and under a gentle vacuum. After the samples were blown to dryness, they were reconstituted to 2 mL in DCM. General scans and high-resolution measurements were being performed on the DCM fractions to analyze the degradation products, and samples were then analyzed by GC/MS as such. The data obtained from experiments were used to calculate the percentage of FF conversion.

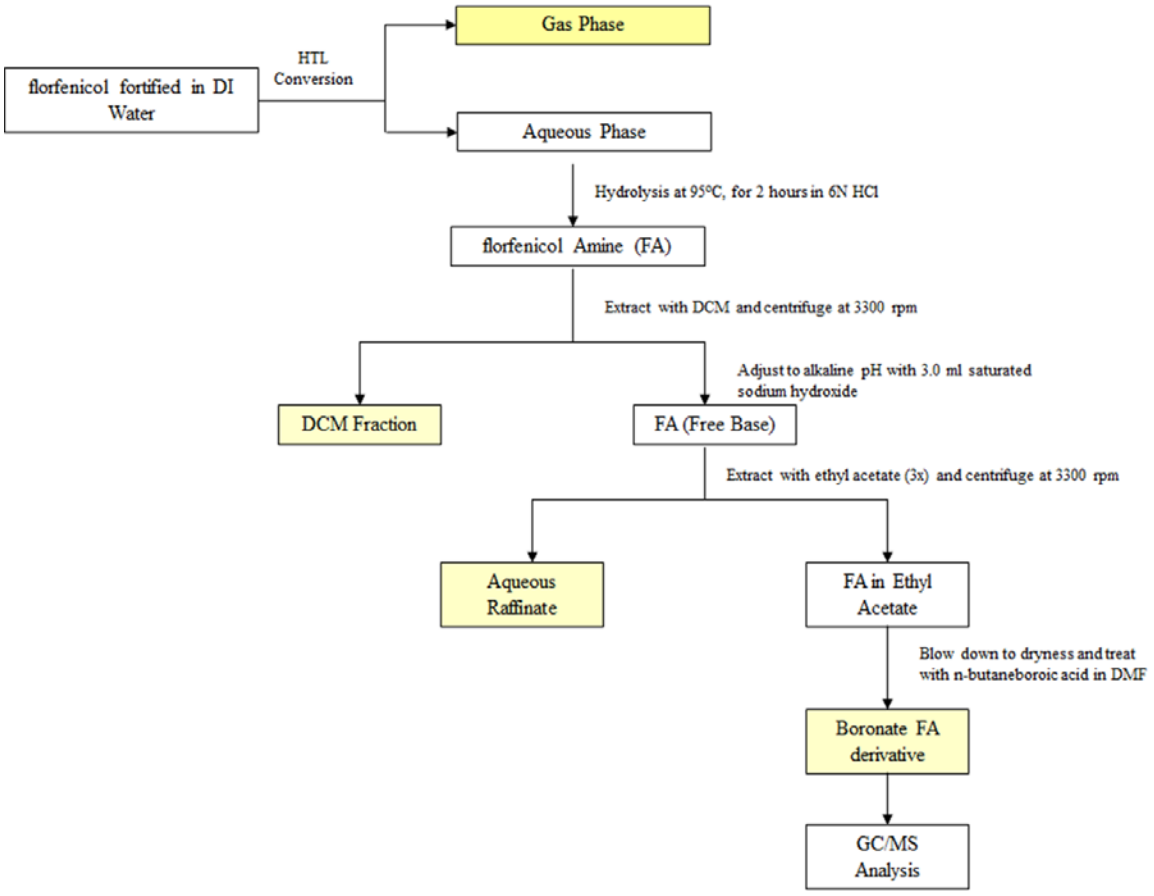


Figure 5.3 Flow diagram of the samples preparation method for florfenicol analysis by GC/MS

5.3 RESULTS AND DISCUSSIONS

5.3.1 ANALYTICAL TECHNIQUE FOR FLORFENICOL BREAKDOWN PRODUCTS

To find the percent conversion of FF to breakdown products, the mmoles of the concentrations in the HTL products were calculated and compared to the mmoles of FF in the DI water. Table 5.3 shows the concentrations of breakdown products in mmoles and the percent conversion to each product. The largest amount of conversion was for FF treated at the higher HTL temperature. The main product was 4-MSB, but small amounts of 4-MSAP and MPS were also

produced. 99.9% of the FF was reduced in HTL product #1; however, only small amounts of breakdown products were present in HTL product #2, so the amount of FF reduced was insignificant.

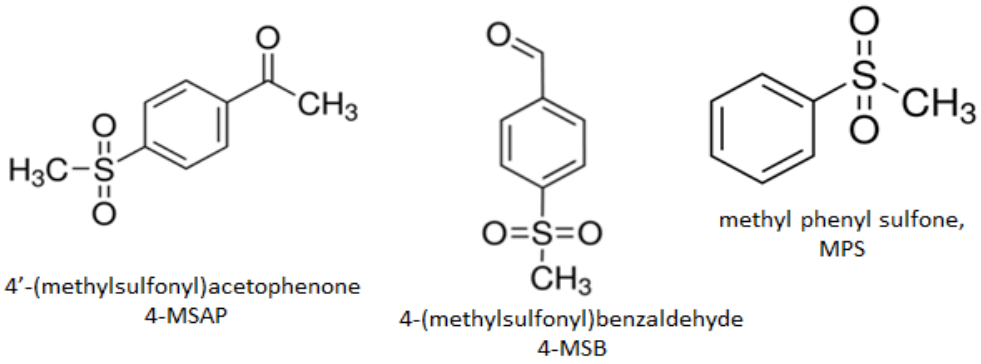


Figure 5.4 Florfenicol breakdown products identified by GC/MS

Table 5.3 Florfenicol percent conversion to breakdown products after HTL tests

	mmole florfenicol spike	mmole 4-MSB	% conversion to 4-MSB
HTL 1 (300°C/30minutes)	0.28	0.16	59%
HTL 2 (300°C/30minutes)	0.28	0.027	10%

	mmole florfenicol spike	mmole 4-MSAP	% conversion to 4-MSAP
HTL 1 (300°C/30minutes)	0.28	0.004	1.40%
HTL 2 (300°C/30minutes)	0.28	<0.003	NA

	mmole florfenicol spike	mmole MPS	% conversion to MPS
HTL 1 (300°C/30minutes)	0.28	0.0041	1.50%
HTL 2 (300°C/30minutes)	0.28	ND	NA

	Sum % conversion to products
HTL 1 (300°C/30minutes)	62%
HTL 2 (300°C/30minutes)	10%

The appropriate reference materials were procured from Sigma-Aldrich and a high-resolution GC/MS method was developed to measure the concentrations of the FF breakdown products. Figure 5.5 (A) presents the chromatograms for measurements of 4-MSAP in the HTL products. The concentration of 4-MSAP in the HTL product #1 was 0.80 mg/L while the concentration of 4-MSAP in the HTL product produced at a lower temperature was less than the lowest calibration standard < 0.5 mg/L. Figure 5.5 (B) presents the chromatograms for measurements of 4-MSB in the HTL products. The concentration of 4-MSB in the HTL product #1 was 30 mg/L while the concentration of 4-MSB in the HTL product produced at a lower temperature was 4.9 mg/L. Figure 5.5 (C) presents the chromatograms for measurements of MPS in the HTL products. The concentration of MPS in the HTL product #1 was 0.64 mg/L while the

concentration of MPS in the HTL product produced at a lower temperature was not detected, <0.5 mg/L.

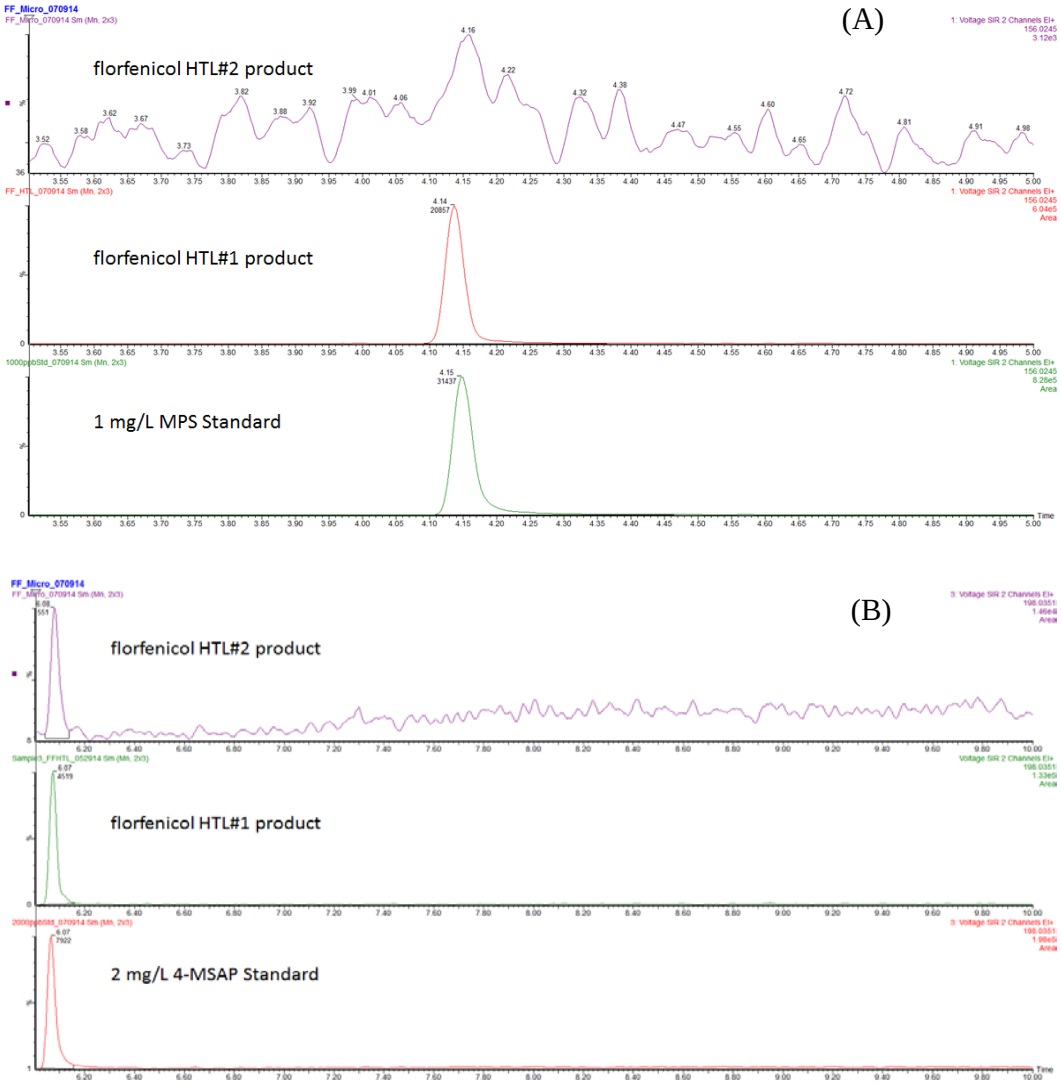


Figure 5.5 (A) Florfenicol percent conversion to MPS after HTL tests (B) Florfenicol percent conversion to 4-MSAP after HTL tests

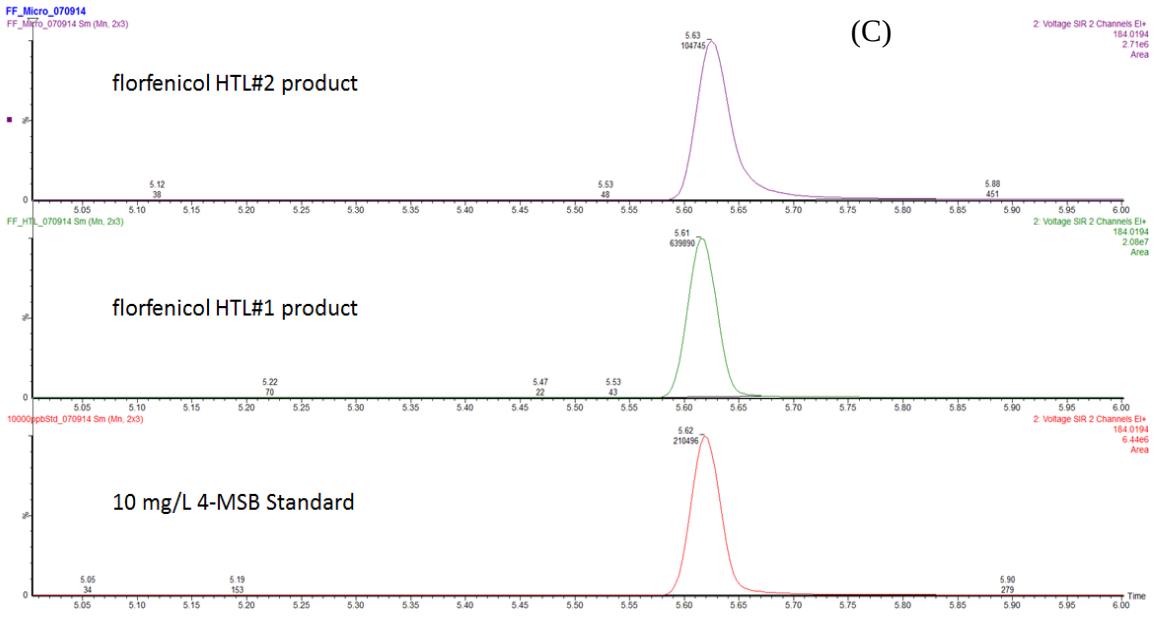


Figure 5.5 (contd.) (C) Florfenicol percent conversion to 4-MSB after HTL tests

5.3.2 EFFECTS OF HTL ON THE FATE OF BIOACTIVE CECS

5.3.2.1 CHARACTERIZATION OF FEEDSTOCK

Table 5.3 displays the residual concentrations of E1, E2, E3, and EE2 in the LPAM, MABB biomass, and HTL-WW produced from HTL under 300°C/60minutes. Residual concentrations of natural estrogenic hormones in the biomass ranged from 19.1 to 377.8 µg/L with serial extraction with methanol based on the method in Chapter 3. After HTL of biomass under 300°C/60minutes, the residual concentrations of hormones ranged from 0.3 to 21.4 µg/L in the HTL-WW. Total and volatile solids content of the biomass was 19.3 ± 0.2% and 81.9%, respectively. Because FF was not used in the SRC, the feedstock for HTL and CHG tests were spiked with FF stock solution and the FF concentration in the feedstock for HTL and CHG was not analyzed.

Table 5.4 Concentrations of baseline hormones and solids content of the LPAM, biomass, and HTL-WW (300°C/60minutes).

	E1	E2	E3	EE2	Total	Total Solids (TS, %)	Volatile solids (% of TS)
LPAM (µg/L)	28.3 ± 0.1	12.4 ± 0.1	30.3 ± 0.5	3.8 ± 0.1	54.8 ± 0.4	3.5 ± 0.4	1.6 ± 0.2
Biomass (µg/L)	375.2 ± 2.5	351.5 ± 52.4	195.5 ± 17.9	19.1 ± 0.1	933.8 ± 18.6	19.3 ± 0.2	81.9 ± 0.04
HTL-WW (µg/L)	3.8 ± 0.1	10.3 ± 0.9	21.2 ± 0.3	0.3 ± 0.03	35.5 ± 1.0	2.5 – 3%	1.5 – 2.5%

5.3.2.2 BIOCRUDE OIL YIELD

Figure 5.6 and Table 5.5 shows the results of experiments regarding the yield of biocrude oil and solid residue for HTL of biomass. The highest production of biocrude oil occurred at 300°C and 60 minutes reaction time, where the biocrude oil yield reached at 40.0% (dry basis), and the solid residue yield was 12.0% (dry basis). As the reaction temperature increased, the yield of biocrude oil increased steadily up to 300°C, and then decreased when the temperature was raised to 350°C. When the biocrude oil yields under 30 and 60 minutes reaction time had compared each other, longer reaction times also generally resulted in higher yield as well.

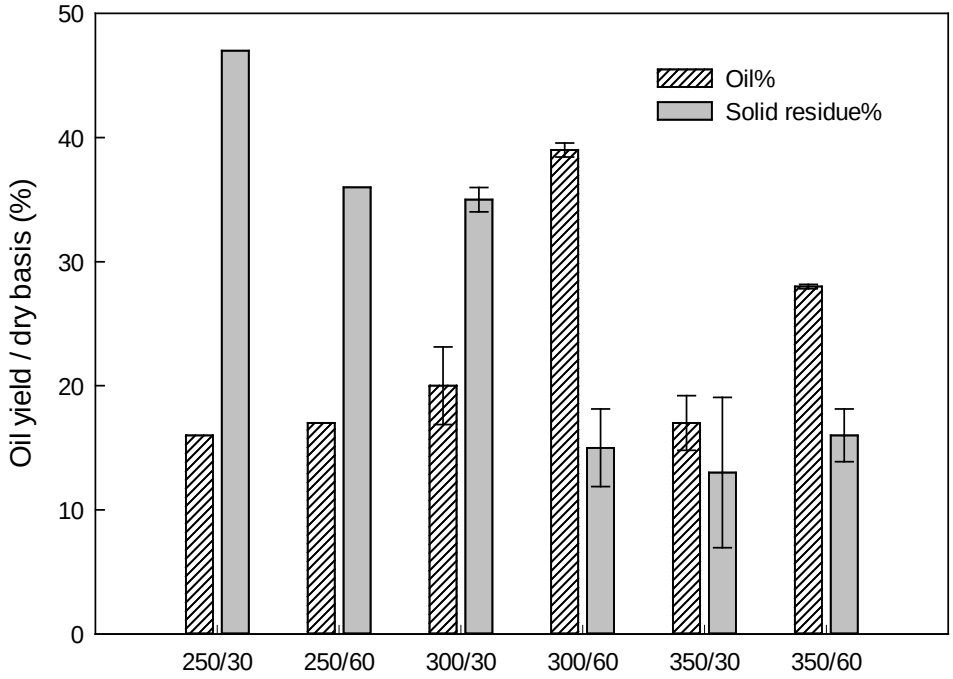


Figure 5.6 Biocrude oil yield and solid residue of biomass under different reaction conditions. The error bars indicate the standard error of the mean

Table 5.5 Biocrude oil yield and solid residue of biomass HTL under different reaction conditions.

Feedstock	Temp (°C)	Time (min)	*Biocrude oil yield (%)	*Solid residue yield (%)	*HTL-WW yield (%)
Mixed algal-bacterial biomass	200	60	4.4	63.1	32.6
	250	30	7.5	47.1	45.4
	250	60	16.7	35.6	47.7
	300	30	19.7	34.1	46.3
	300	60	40.0	12.0	47.9
	350	30	17.3	32.4	50.4
	350	60	27.6	24.8	47.6

* Calculated based on the weight of total biomass

5.3.2.3 EFFECTS OF REACTION TEMPERATURE ON CECS REMOVAL

(A) Estrogenic hormones

Based on the residual and spiked concentrations (E1, E2, E3, and EE2) in the biomass, percent removal of total estrogenic hormones was calculated for all the HTL conditions. Figure 5.7 shows that the removal of total estrogens at 300°C/60minutes was higher than the percent removal of the other HTL conditions, which followed the same trend as the results of biocrude oil yield. However, there was no significant relationship between 300°C/60minutes and the other conditions for the removal of hormones. Overall percent removal of E3 was 83.5% on average, which was lower than the percent removal of other compounds because the E1, E2, and EE2 could be transformed to the E3 during the hydrothermal reaction assuming that it follows the previously reported transformation pathways for anaerobic conditions (Hutchins et al., 2007). Another previous study showed that E3 was one of main products of estrogen degradation under anaerobic conditions and this could explain the lower degree of removal for E3 in our study (Zheng et al., 2012).

In conclusion, the best operating conditions for HTL processes to provide simultaneous bioenergy production and CECs removal was 300°C/60minutes, which ranged from 82% to 98.4% for the percent removal of estrogenic hormones from the spiked biomass. The E3 (82%) removal percentage was lower than the percent removal of any other compounds, which can be explained by transformation or breakdown of other hormones to E3, according to the pathways for anaerobic conditions in previous studies. Higher temperatures at 60 minutes for HTL tests provided more effective removal of E1 and E3 because the percent removal was increased from 91% to 97% and from 81% to 86%, respectively. However, the optimal operating condition of HTL for bioenergy

production (300°C/60minutes) simultaneously resulted in the highest removal of total estrogenic hormones.

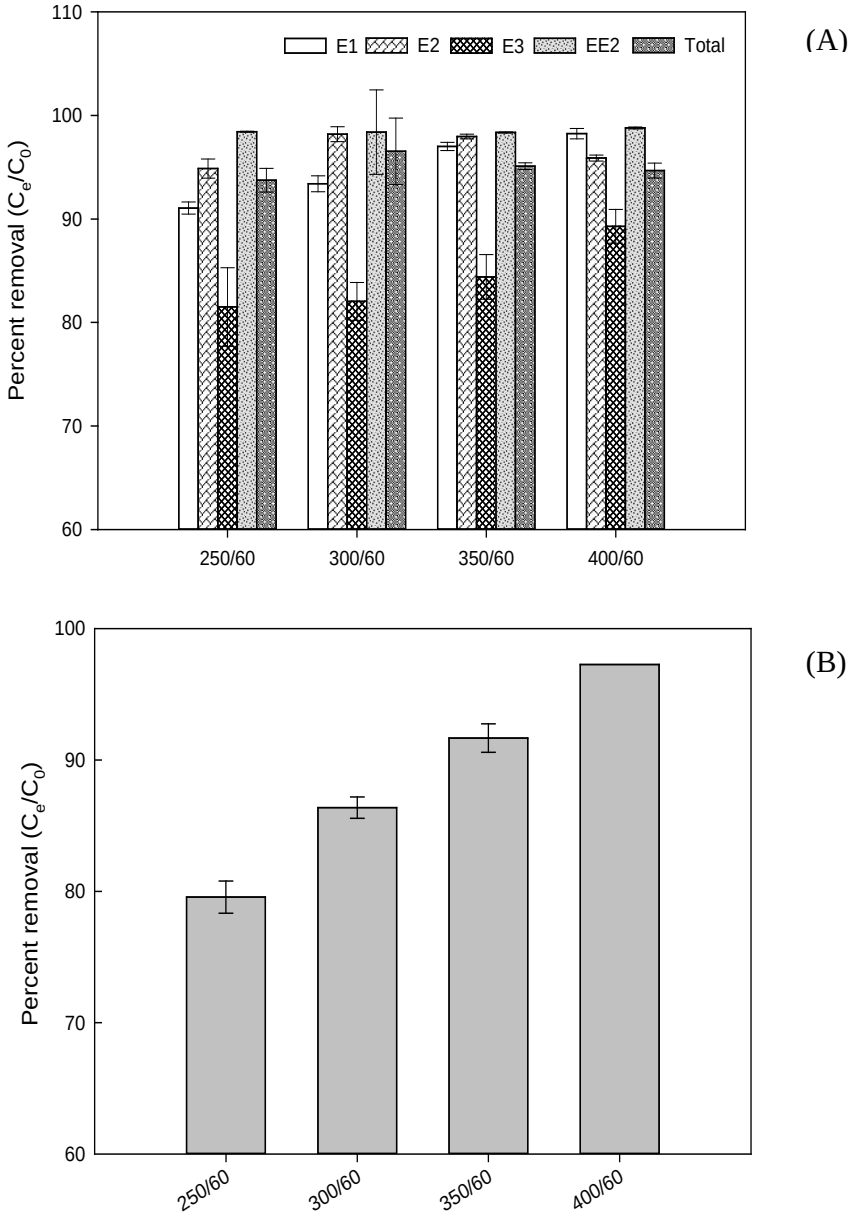


Figure 5.7 (A) Effects of different reaction temperature on the removal of E1, E2, E3 and EE2 in HTL tests (n ⩾3) (B) Effects of different reaction temperature on the removal of FF in HTL tests (n⩾2). The error bars indicate the standard error of the mean

(B) FF and its breakdown products

The removal of FF during CHG tests enhanced from 79.6% to 97.3% as the reaction temperature was increased from 250 to 400°C. This data is displayed on Figure 5.7 (B), which presents other FF removal data during HTL at different reaction temperatures. CHG of biomass at 400°C and 60 minutes reaction time saw the highest FF removal, and could also produce the highest oil yield based on Table 5.5. When the temperature was increased from 250°C to 400°C, FF removals also generally increased. At 400°C, the concentrations were below the GC/MS analysis limit of detection and so, there was no error bar in the data. As such, we could infer that the reaction temperatures in HTL tests on biomass are positively related to the removal of FF. Thus, the difference of FF removal between 250°C and 400°C was significant (P=0.04) that could reject the null hypothesis.

The optimal operating condition for HTL processes to simultaneously remove FF and FF breakdown products in HTL-WW was at 400°C/60minutes, which resulted in FF percent removals of 97.3% and total distribution of FB to HTL-WW of 34.1%. However, Table 5.7 demonstrated an insignificant difference of percent distribution of FB during HTL under different reaction temperature (P=0.68) and time (P=0.29).

5.3.2.4 EFFECTS OF REACTION TIME ON CECS REMOVAL

(A) Estrogenic hormones

After the HTL tests at 30 and 60 minutes under 300°C, the removal of E1, E2, E3, EE2, and total hormones in the HTL-WW were analyzed in Figure 5.8 (A). The increased percent removal of estrogenic hormones after 60 minutes of HTL tests ranged from -5.8% to 18.9%, which demonstrates that the hormones removal at 60 minutes were higher than the results at 30 minutes

of HTL tests except E3. As mentioned in Chapter 4, E3 percent removal can be decreased or negative due to increased concentration of E3 after the transformation from E2, EE2, and E1 in the HTL reaction. According to a technical report by Dan Knorr, (2013) on HTL system processing of biomass, the major energy input for HTL was the energy used for heating the reactor and was proportional to the total heating duration and designated reaction temperature, respectively. Therefore, longer hydrothermal reactions could provide more hydrothermal energy for physicochemical reactions, and subsequently, the bio crude oil yield and percent removal of estrogenic compounds can be increased.

(B) FF and its breakdown products

The percent removal of FF and FF breakdown products in HTL-WW were analyzed during biomass HTL with different reaction time (30, 60, and 90 minutes under 300°C), and the FF removal were increased from 73.6 to 96.8% in Figure 5.8 (B). The enhanced percent removal of FF with increasing reaction time during HTL demonstrates that FF removal was sensitive to reaction time, and the statistical difference of FF removal between 30 and 90 minutes was moderate (P=0.06). This observation suggested that when the MABB biomass was used for HTL tests, increasing in reaction time and temperature would potentially increase the removal of FF. Increasing reaction temperature and time, however, can also enhance the yield of bio-crude oil. Therefore, it is important to balance between optimizing the process yield and minimizing the environmental impact of process. Dan Knorr, (2013) reported that total energy input could be proportional to the reaction time and temperature in HTL, and higher removal of CECs and yield of biocrude oil could be expected due to the more hydrothermal energy.

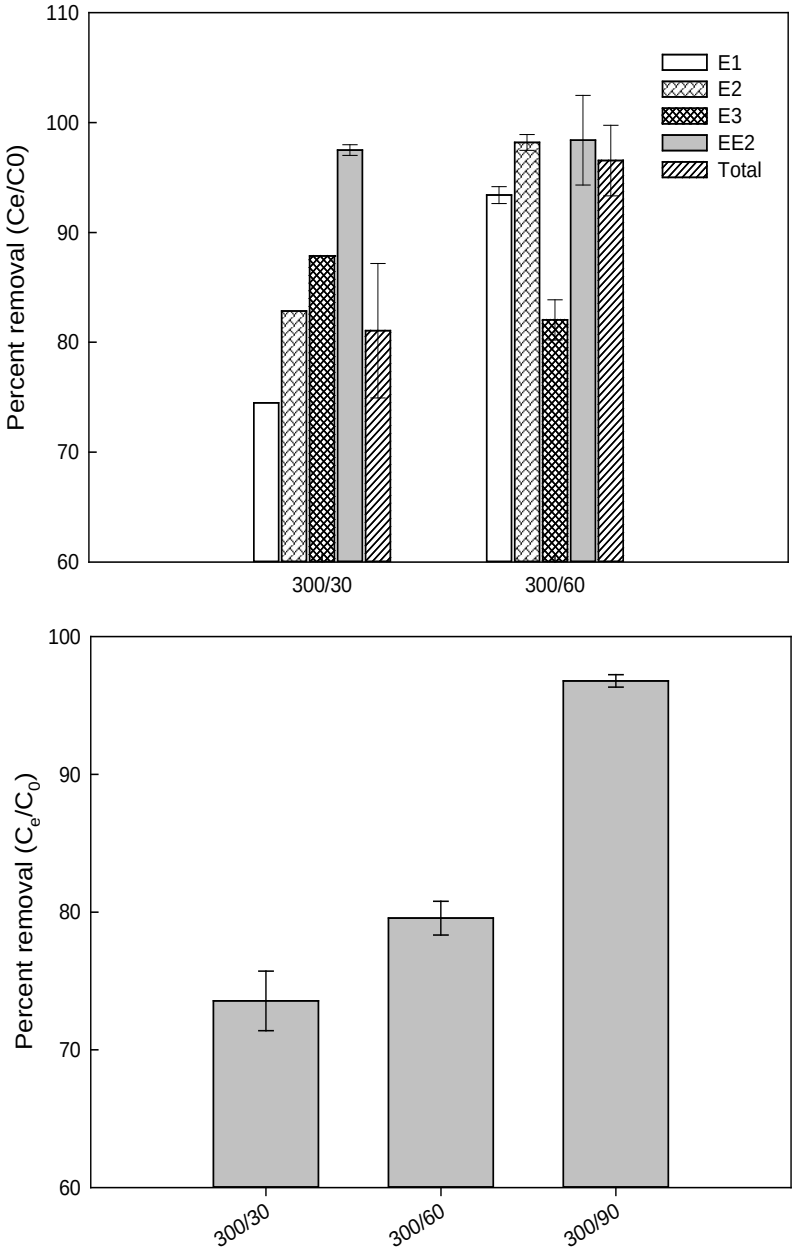


Figure 5.8 (A) Effects of different reaction time on the removal of E1, E2, E3 and EE2 in HTL tests (n⩾2) (B) Effects of different reaction time on the removal of FF in HTL tests (n⩾2). The error bars indicate the standard error of the mean

5.3.3 CATALYTIC HYDROTHERMAL GASIFICATION

5.3.3.1 GAS AND ENERGY YIELD OF BIOMASS CHG

To investigate the gas production and energy recovery of biomass CHG, direct catalytic hydrothermal gasification of cultivated biomass was conducted at three selected conditions. Table 5.6 shows the results about the gas and energy yield for CHG tests of the biomass. As the reaction temperature was increased from 400 to 600°C, the gas yield from CHG tests with biomass feedstock were enhanced from 39.3 to 63.6% based on the dried weight of biomass. The highest gas production occurred at 600°C and 60 minutes reaction time with Ruthenium catalyst, where the biocrude oil yield reached at 1.0% (dry basis) and the yield of effluent wastewater after CHG conversion (CHG-WW) was 36.1% (dry basis).

5.3.3.2 EFFECTS OF REACTION TEMPERATURE ON CECS REMOVAL

(A) Estrogenic hormones

Figure 5.9 (A) presents the removal of E1, E2, EE2, and E3 during CHG with a variety of temperature conditions. Removals generally increased with the temperature from 350°C to 450°C, and then plateaued between 96.5% to 99.9% with higher temperatures, because all the compounds were below the limit of detection by our GC/MS analysis. Essentially complete removal of the hormones was observed at CHG temperatures of 450 °C or higher. When the water temperature is above the critical point (temperature: 374°C, pressure: 22.1 MPa) in CHG tests, the physical properties of water change significantly, and this supercritical water has extremely good mass transport properties and facilitate the breakdown of organics to produce gases such as H_2, CO, CO_2, and CH_4 (Krishnan et al., 2016). These physico-chemical reactions effectively break down the estrogenic hormones as well. Therefore, temperatures above the critical point can maximize

the percent removal of estrogenic hormones such as E1, E2, E3, and EE2 in CHG of spiked biomass. Thus, there was no significant relationship between all the conditions in CHG for hormones removal.

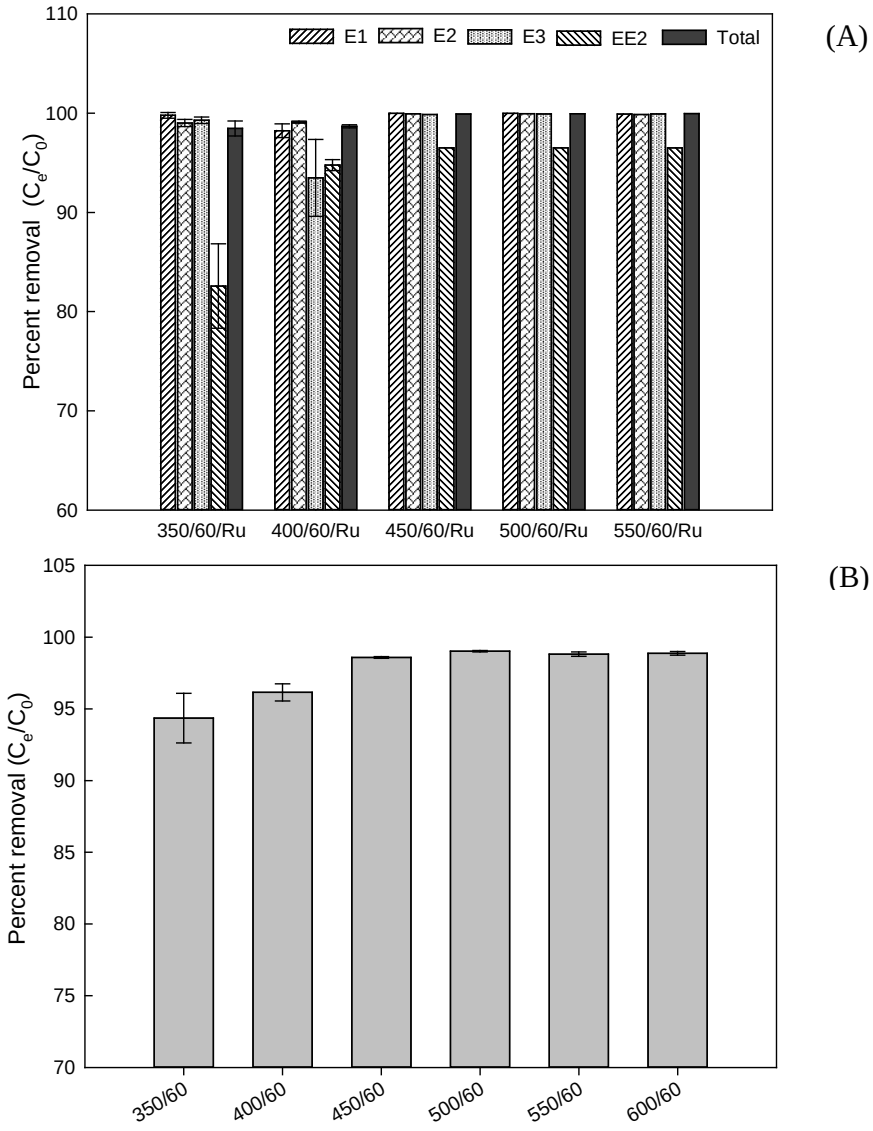


Figure 5.9 (A) Effects of reaction temperature on the fate of estrogenic hormones in CHG (n⩾2) (B) Effects of reaction temperature on the fate of FF in CHG (n⩾2). The error bars indicate the standard error of the mean

Table 5.6 Gas and energy yield for CHG of the biomass

Feedstock	Temperature (°C)	Time (minute)	[a]CHG-WW yield (%)	[a]Solid residue yield (%)	[a]Biocrude oil yield (%)	[a]Gas yield (%)	[b]Gas composition (%)				[c]Energy recovery (%)
							H_2	CH_4	CO_2	CO	
Mixed algal-bacterial biomass	400	60	21.3%	24.8%	14.5%	39.3%	9.0%	5.2%	19.1%	NA	7.8%
	500	60	39.1%	[d]0.0%	1.4%	60.3%	5.9%	42.6%	28.9%	4.9%	76.9%
	600	60	36.1%	[d]0.0%	1.0%	63.6%	5.6%	36.6%	28.6%	6.0%	63.6%

a. Calculated based on the weight of total biomass

b. Calculated based on the volume of total gas production

c. Calculated based on the weight of dried biomass

d. Due to some loss during transfer and sample handling

(B) FF and FF breakdown products

The percent removal of FF and the distribution of FF-BP to CHG-WW were determined based on the spiked concentration of FF in the biomass and residual concentration of FF-BP in the CHG-WW. Although FF removal was gradually enhanced as the temperature increased from 350°C to 450°C, it plateaued to between 98.6% to 99.0% at temperatures higher than 450°C. As shown in Figure 5.9 (B), CHG temperatures of 450 °C or higher saw the highest removal of the FF, following the trend of hormones removal in Figure 5.9 (A). To evaluate the correlation between FF removal and reaction temperature in CHG, a Pearson correlation analysis was conducted and there was a significant relationship between FF removal and the reaction temperature ($r=0.86$, $P=0.028$) in CHG.

Also, the gas yield increased from 39.3% to 60.3% as the reaction temperature was increased from 400°C to 500°C. Therefore, the removal of bioactive CECs was positively related to the reaction temperature in biomass CHG, and the highest FF removal rate and gas yield in biomass CHG tests could be obtained at reaction temperatures higher than 450°C. The biomass feedstock provided a smaller protective effect with FF than with estrogenic hormones. This can most likely be explained by the higher water solubility (1.32 mg/mL) and the lower log Kow (0.37) of FF, which sorption to biomass is unfavored to being in the water for FF (Table 3.1). The percent removal of FF was higher than 91% when the CHG process was operated at 350°C, which suggests that increasing the reaction temperature could overcome any protective effect provided by the presence of feedstock (Figure 5.9 B). Removal of FF with HTL at 350°C and reaction times of 60 minutes or more was observed to near or below detection limits.

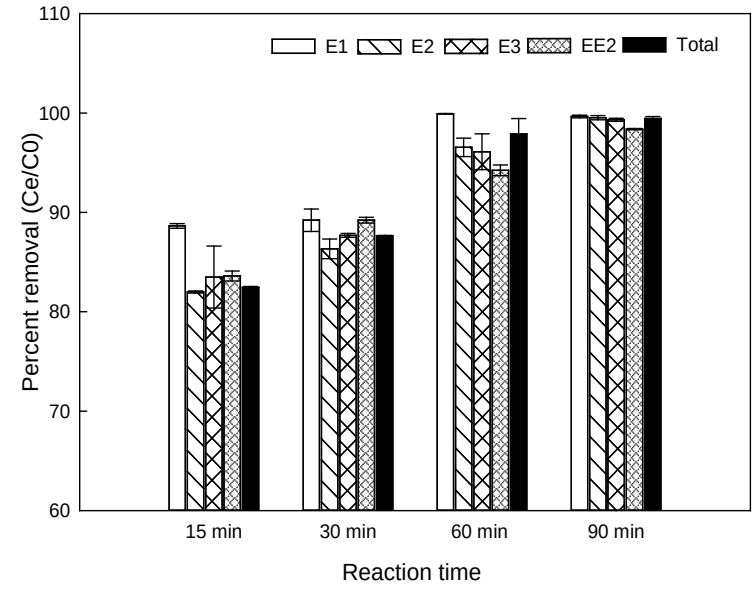
According to Table 5.7, percent distribution of each FF-BP such as MPS, 4-MSB, and 4-MSAP ranged from 0.1 to 11.5% based on the original amount of FF for spiking biomass. The

relationship between FF-BP distribution and increasing reaction temperatures (r= -0.87, P=0.02) was negative linear correlation between reaction temperature and total FF-BP distribution to CHG-WW, which means the percent distribution of FF-BP falls with increasing reaction temperature in CHG tests. In conclusion, simultaneous bioenergy production and removal of FF by CHG processes were optimal at higher than 450°C in CHG, which resulted in the 99.9% and 98.9% removal of total hormones and FF from the spiked biomass, respectively. Thus, FF-BP in CHG-WW had a negative linear relationship with reaction temperature, and it was expected that CHG-WW with higher reaction temperatures would have lower concentrations of FF-BP.

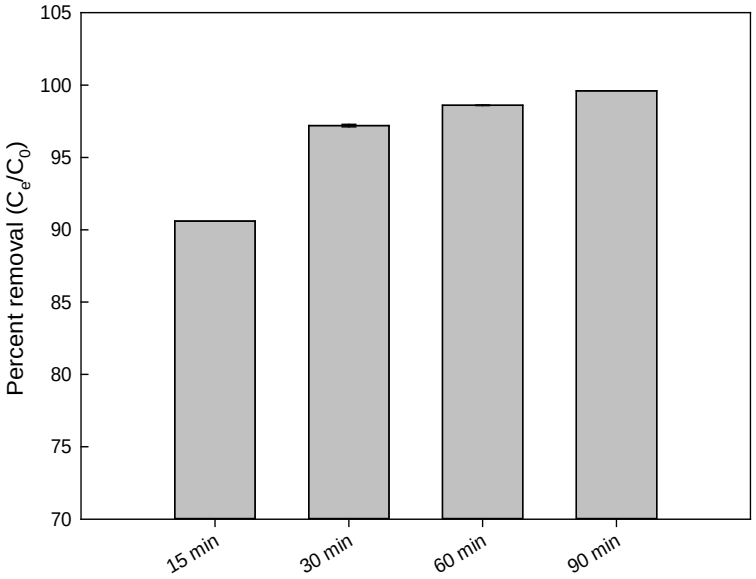
5.3.3.3 EFFECTS OF REACTION TIME ON CECS REMOVAL

(A) Estrogenic hormones

The effects of the reaction time on the fate of CECs under CHG were explored by using the increasing reaction time for biomass CHG under 350˚C/Ru/10g biomass, because reaction time is one of the most critical parameters which can control the total heating energy and reaction kinetics for higher gas yield (Lu et al., 2012; Susanti et al., 2011). The removal of estrogenic hormones ranged from 98.4% to 99.7%, that were proportional to the increased reaction times because longer reaction times can provide more thermal energy for the breakdown of estrogenic compounds during biomass conversion in CHG (r=0.95, P=0.04). Thus, percent removal of total hormones after 90 minutes of CHG ranged from 99.3% to 99.7%, which equates to the removal of almost all hormones. In conclusion, removal of estrogenic hormones was elevated with increased reaction temperatures (~ 99.7%), and hormones removal had positive and significant relationship with reaction time (P<0.05).



(A)



(B)

Figure 5.10 (A) Effects of reaction time on the fate of estrogenic hormones in CHG under 350° C/Ru/10g biomass (n≥4) (B) Effects of reaction time on the fate of FF in CHG under 350° C/Ru/10g biomass (n≥2). The error bars indicate the standard error of the mean

Table 5.7 Summary of removal of FF and FF breakdown products at hydrothermal liquefaction and catalytic hydrothermal gasification

		FF removal (%)	FF breakdown products in aqueous phase (%)			
			MPS	4-MSB	4-MSAP	Total
Hydrothermal liquefaction (HTL)						
Temperature (°C)	250	79.6 ± 1.2	0.6	5.4	27.2	33.2
	300	87.1 ± 0.8	0.6	0.9	21.8	23.3
	350	91.7 ± 1.1	0.6	5.4	27.2	33.2
	400	97.3	1.5	5.4	27.2	34.1
Time (minute)	30	73.6 ± 2.2	6.1	27.2	164.0	197.3
	60	87.1 ± 0.8	0.6	0.9	21.8	23.3
	90	96.8 ± 0.5	0.2	1.9	8.0	10.1
Catalytic hydrothermal gasification (CHG)						
Temperature (°C)	350	94.4 ± 1.7	0.9	1.8	11.5	14.2
	400	96.1 ± 0.6	0.3	1.8	8.8	10.9
	450	98.6 ± 0.1	1.9	0.9	6.9	9.7
	500	99.0 ± 0.1	0.1	0.9	4.4	5.4
	550	98.8 ± 0.2	0.1	0.9	4.4	5.4
	600	98.9 ± 0.1	0.8	1.0	5.2	7
Time (minute)	15	99.6	0.1	0.4	2.0	2.5
	30	97.2 ± 0.1	0.6	0.7	4.7	6.0
	60	98.6 ± 0.1	0.9	1.8	11.5	14.2
	90	99.6	1.7	0.4	3.7	5.9
Catalyst type	Ru	96.1 ± 0.6	0.1	1.2	5.8	7.1
	Ra-Ni	99.0 ± 0.3	0.1	1	4.8	5.9
	Ru + NaOH	97.4 ± 0.2	0.2	2.2	11	13.4
	Ra-Ni + NaOH	99.2	0.1	0.7	3.3	4.1
	NaOH	94.1 ± 2.5	0.6	5.9	29.6	36.1
Catalyst amount (g/10g biomass)	0.1	94.5 ± 0.7	0.0	0.4	8.6	9
	0.5	98.7	0.9	1.8	11.5	14.2
	1	94.8 ± 2.6	0.2	2.2	37.3	39.7
	2	88.9 ± 2.0	4.2	1.3	9	14.5
	4	98.4 ± 0.3	0.0	0.4	2.2	2.6
Sequential HTL & CHG						
Temperature (°C)	450	95.3 ± 0.4	0.1	0.1	0.5	0.7
	500	96.5 ± 0.7	0	0.2	0.8	1
	550	97.1 ± 0.3	0	0.1	0.5	0.6
	600	98.7 ± 0.4	0	0.1	0.5	0.6
Time (minute)	30	93.2 ± 0.2	0	0.1	0.5	0.6
	60	96.5 ± 0.7	0	0.2	0.8	1
	90	98.2 ± 0.7	0	0.1	0.5	0.6

(B) FF and its breakdown products

Reaction time was gradually increased from 15 to 90 minutes for each CHG of biomass test to study the effects of reaction time on the removal of FF and distribution of FF-BP in CHG-WW. Figure 5.10 (B) displays the removal of FF which plateaued to 98.4% on average as the reaction time increased to 30 minutes or more when CHG was performed at 400°C with a Ru catalyst. For example, as the reaction time increased from 15 to 30 minutes, the removal of FF increased from 90.6 to 97.2%, supporting the idea that a positive effect on the breakdown of this bioactive compound occurred as the reaction time was increased But, the difference in the FF removal longer than 30 minutes was insignificant that supported the stabilization of FF removal or complete removal of FF from biomass ($r=0.83$, $P=0.17$).

The FF-BP in CHG-WW generally increased from 15 to 60 minutes, and then decreased at 90 minutes as the CHG reaction time increased. More specifically, the reaction time of the CHG was increased from 15 to 90 minutes and the subsequent distribution of FF-BP in CHG-WW fluctuated from 2.5 to 14.2%, and then to 5.2%, which means that it was hard to find the relationship between reaction time and FF-BP in CHG-WW because the FF-BP could be eliminated or distributed to aqueous phase during the biomass CHG.

5.3.3.4 EFFECTS OF CATALYST TYPE ON CECS REMOVAL

(A) Estrogenic hormones

To investigate the effects of catalyst type on the fate of CECs, fiver different combinations of catalysts including Ru, Ra-Ni, and NaOH, were used for the biomass CHG to find the most effective catalyst to remove estrogenic hormones. 0.5g of each catalyst was homogenously mixed with 10g of biomass, and the baseline operating condition of CHG was 400˚C/60minutes. After

CHG tests, Ra-Ni/NaOH showed slightly better percent removal of total hormones than 5 different catalysts, and the performance of catalysts for total hormones removal follows in this order; Ra-Ni/NaOH (99.2%) > Ra-Ni (99.0%) > Ru/NaOH (97.4%) > Ru (96.1%) > NaOH (94%). After adding NaOH to the metal catalyst experiments, percent removal of total hormones for the Ru and Ra-Ni increased by 0.2% and 1.3%, respectively. However, the NaOH demonstrated effective removal (94%) of total hormones even in the absence of the metal catalyst. These results indicate that the performance of NaOH by itself as substitute for a metal catalyst to remove estrogenic hormones was promising, but it could enhance the hormone removal when used in combination with the Ra-Ni.

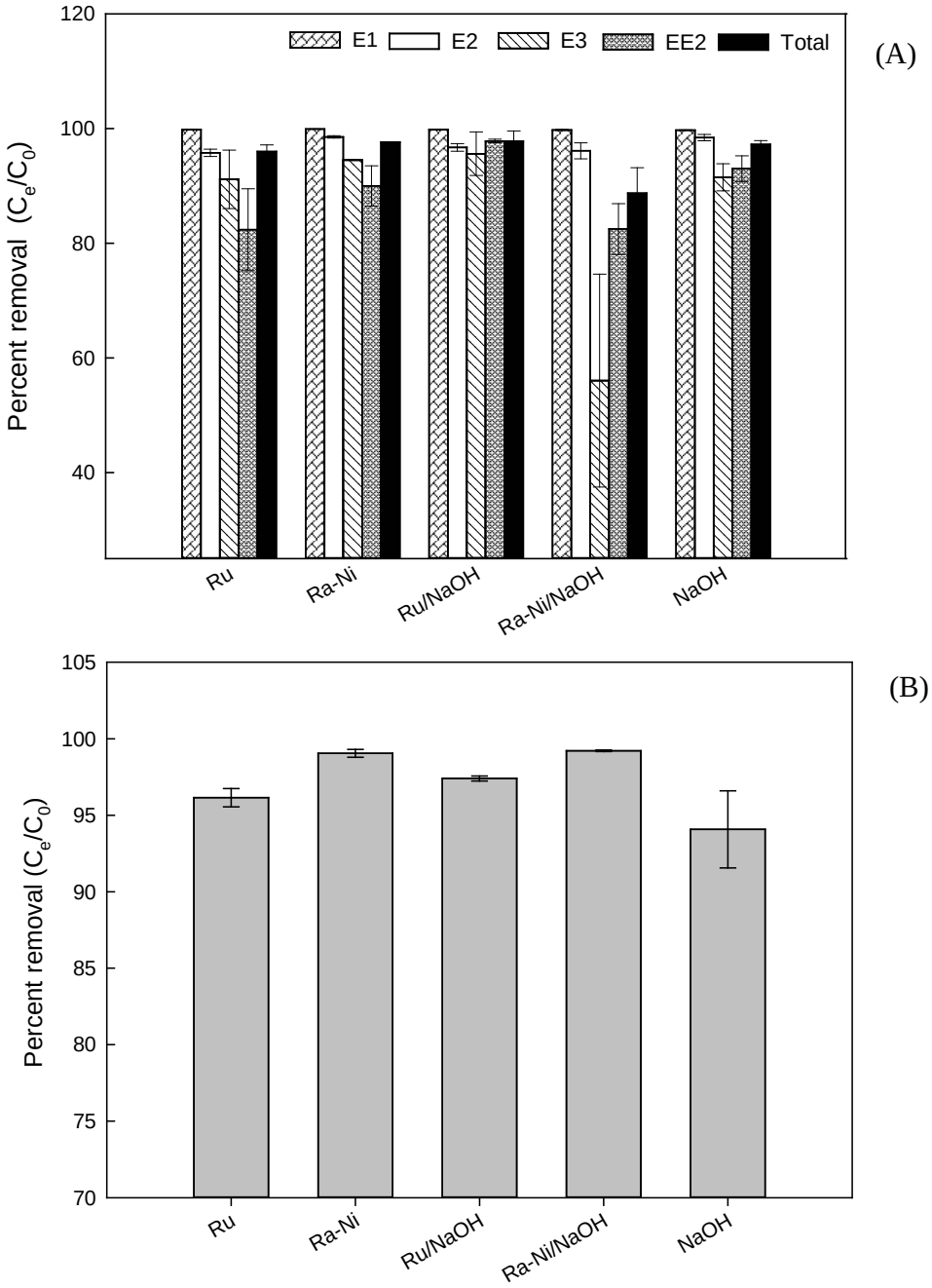


Figure 5.11 (A) Effects of catalyst type on the fate of estrogenic hormones in biomass CHG (n⩾4) (B) Effects of catalyst type on the fate of FF in biomass CHG (n⩾2). The error bars indicate the standard error of the mean

(B) FF and its breakdown products

To investigate the effects of catalyst type on the fate of FF and FF-BP under CHG, fiver different combinations of catalysts including Ru, Ra-Ni, and NaOH, were used for the biomass CHG to find the most effective catalyst to remove FF. In Figure 5.11 (B), FF removal during the biomass CHG with different catalyst ranged from 94.1 to 99.0%, and Ru and/or Ru/NaOH demonstrated the highest FF removal (99%) among the 5 different combinations of catalysts. However, the FF removal was basically higher than 94.1% with any types of catalyst, which demonstrated that CHG was effective to remove artificially spiked FF in biomass because FF is water soluble and thus, it is more accessible during the CHG treatment.

According to the types of catalyst, the percent distribution of 4-MSAP in CHG-WW ranged from 3.3 to 29.6% as depicted in Table 5.7. The highest distribution of three FF-BP to CHG-WW was by NaOH. More than 94.1% of FF can be removed by CHG with biomass, but the lowest FF removal, compared to other catalysts, was by the NaOH which increased the distribution of FF-BP to the aqueous phase after CHG tests. Although, no antibiotic resistance capacity was shown by FF-BP, during the selection of catalysts, CHG tests could exclude the NaOH catalyst due to lower FF removal and higher FF-BP in CHG-WW.

5.3.3.5 EFFECTS OF CATALYST AMOUNT ON CECS REMOVAL

(A) Estrogenic hormones

The effects of the catalyst quantity on the fate of hormones were explored by using the different quantities of the Ru catalyst during biomass CHG under 350˚C/60minutes. As the catalyst dose was increased from 0.1 to 4g per 10g biomass, percent removal of estrogenic hormones in the CHG-WW were gradually increased and stabilized to between 98.1% and 99.2% (Figure 5.12

A). However, percent removal of EE2 positively enhanced with increasing catalyst amounts (r=0.87, P=0.05), and the results stated that the increased quantities of catalysts in CHG tests could enhance the removal of estrogenic hormones from the biomass, and especially significant for EE2 removal. However, due to the high price of catalysts ($ 21.9/g Ru, Sigma Aldrich) and characteristics of metal catalysts that can absorb some portion of the water in CHG runs, the optimal dose of catalyst has yet to be found through these tests, even though hormones removal reached to the stable results when using 1g of the Ru catalyst (100 mg Ru/g biomass).

In conclusion, percent removal of estrogenic hormones in the biomass CHG was sensitive to the quantities of catalyst and increased based on the higher doses of the Ru catalyst, but not significant except EE2. Adding a catalyst equal to 10% of the total biomass feedstock could be the optimal dose of Ru catalyst in the CHG tests under 350˚C/60minutes, if the energy recoveries are analyzed with different amount of catalyst.

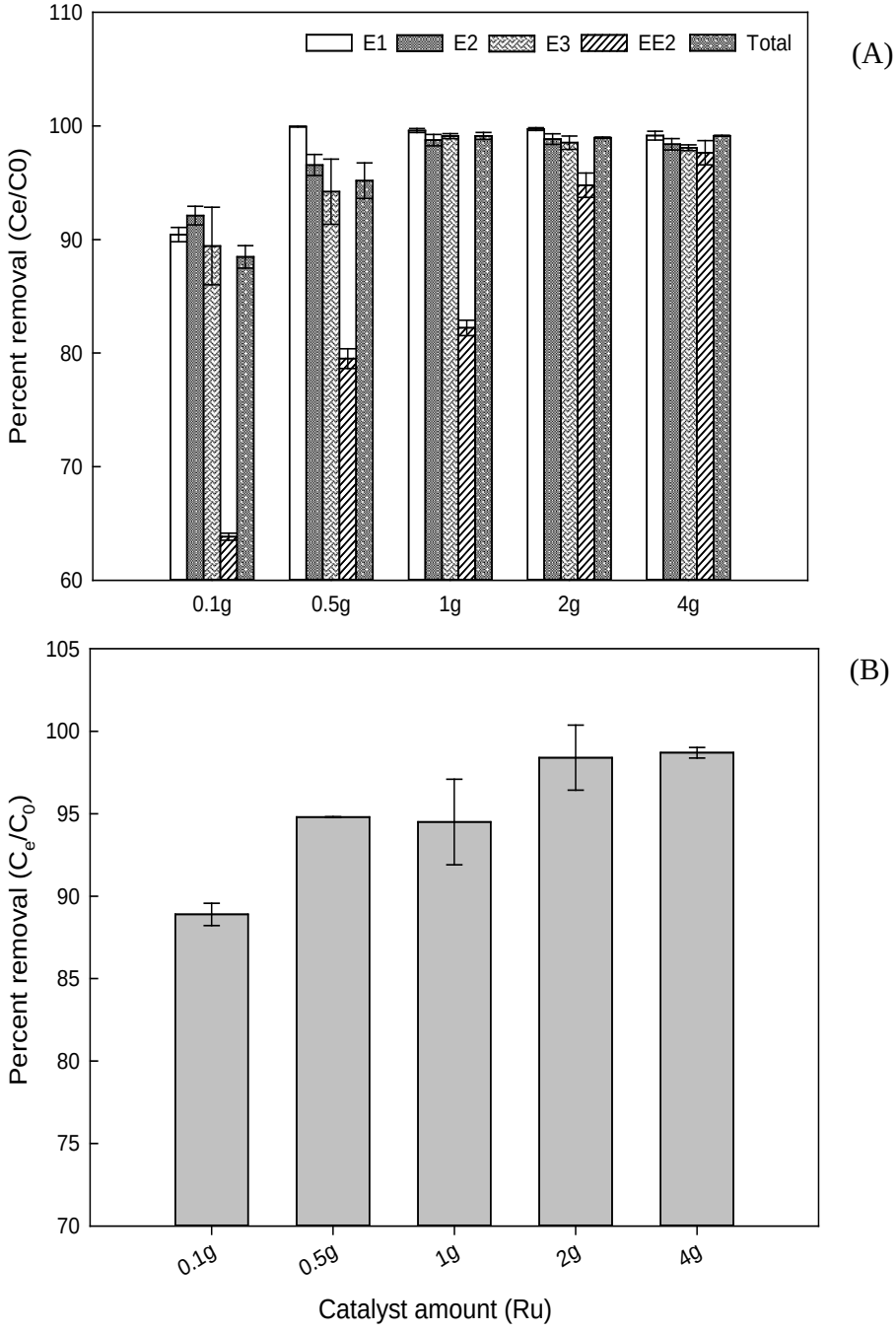


Figure 5.12 (A) Effects of catalyst amount on the fate of estrogenic hormones in CHG (n≥2) (B) Effects of catalyst amount on the fate of FF in CHG under 350° C/Ru/10g biomass (n≥2). The error bars indicate the standard error of the mean

(B) FF and its breakdown products

In the presence of various quantities of Ru catalyst, Figure 5.12 (B) and Table 5.7 present the percent removal of FF from biomass and distribution of FF-BP to CHG-WW. The FF removal was increased from 88.9% to 98.7% and stabilized to around 98.6% as the catalyst dose in CHG was increased from 0.1 to 4g per 10g biomass (r=0.1, P=0.87). However, percent distribution of FF-BP to CHG-WW gradually decreased from 39.7% to 2.6% with increasing amounts of the catalyst, which states that higher doses of catalyst removed most of the FF-BP from the CHG-WW (r=-0.39, P=0.51). These results demonstrated that the increased quantities of catalysts in the biomass CHG could enhance the removal of FF and FF-BP from the biomass and CHG-WW, respectively, but there was no statistical relationship (Table 5.9).

As we stated in the previous results of hormones, the optimal dose of Ru catalyst could be decided based on the catalyst price and the removal efficiency of CECs from the biomass feedstock. Therefore, adding a catalyst equal to 10% of the total biomass feedstock could be the optimal dose of Ru catalyst in the CHG tests due to the relatively high removal of CECs at 1g dose of catalyst and lower cost compared to the higher dose.

5.3.4 SEQUENTIAL HTL AND CHG OF BIOMASS

In Figure 5.13 (A), the removal of FF was enhanced from 95.3% to 98.7% with increasing reaction temperatures from 450°C to 600°C, which corresponded to a positively related significant relationship (r=0.99, P=0.01). HTL/CHG tests with increasing reaction time were analyzed for the removal of FF and distribution of FF-BP in the HTL/CHG-WW (Figure 5.13 B). FF removal saw enhancements from 93.2% to 98.2% with longer reaction time, which demonstrated that increased reaction time has a positive relationship with FF removal, but was insignificant (r=0.98, P=0.12).

Finally, the distribution of FF-BP to HTL/CHG-WW did not show any pattern, which means no correlation between FF removal and reaction parameters such as reaction temperature (r=0.0, P=NA) or time (r=-0.48, P=0.52).

As shown in previous results, the required energy was proportional to the heating duration and designated reaction temperature, respectively. Subsequently, the hydrothermal energy required for physicochemical reactions could be fueled by extended hydrothermal reactions, and the FF removal as well as the energy recovery could be increased (Table 5.6).

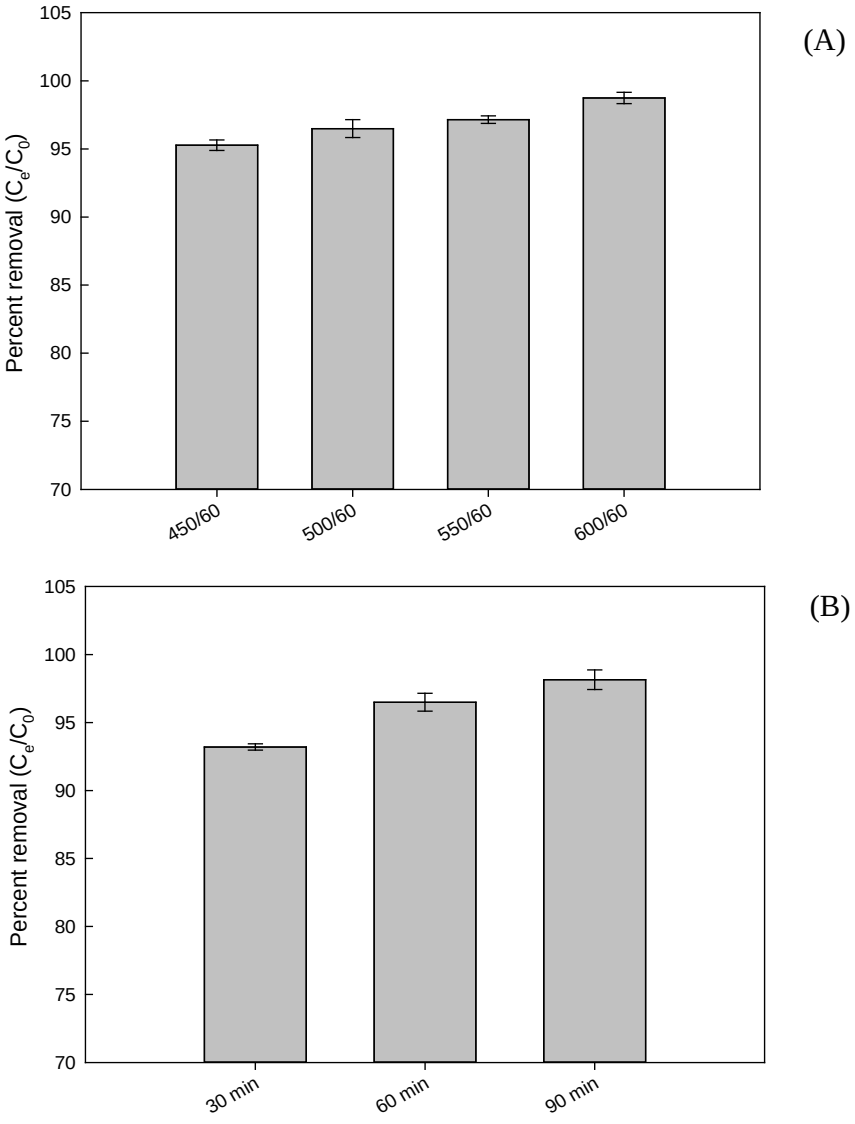


Figure 5.13 (A) Effects of reaction temperature and (B) reaction time on the fate of FF during sequential HTL/CHG tests. The error bars indicate the standard error of the mean

5.3.5 YES YEAST CELL ASSAY FOR ESTROGENIC ACTIVITY

5.3.5.1 ESTROGENIC ACTIVITY IN HTL-WW

A suitable concentration response could be interpolated in a reference compound E2 dose-response curve to determine the estrogenic potency of samples. Estrogenic potency of a compound

or extract in a sample was labeled as EEQ and this was usually expressed relative to E2 at the EC_{50}, where the highest estrogenic effect was detected 50% of the time.

Based on the procedure in Chapter 3 for liquid samples, residual E1, E2, E3 and EE2 from the HTL-WW was purified after being extracted. The Xenoscreen YES cell assay was used to quantify the estrogenic activity of HTL-WW under various operating conditions of HTL. Finally, the residual concentrations of hormones and estrogenicity assay will be used to evaluate the efficiency of the integrated manure management system in removing estrogenic CECs. The EC_{50} value of an E2 standard curve was shown to reside in the microgram per liter range (EC_{50}: 0.11µg/L).

Figure 5.14 (A) showed the agonistic response of the yeast to HTL-WW with various reaction temperatures. The EC_{50} of 250, 300, 350, and 400°C were 0.19, 0.46, 1.11, and 1.21µg/L, respectively. According to the EC_{50} values, the 250°C reaction temperature had the lowest EC_{50} value and was the most potent among the tested samples, and other EC_{50} values gradually increased. This indicates that higher temperatures in the HTL of biomass markedly reduces the estrogenic activity of HTL-WW, which shows a similar trend to the removal of estrogenic hormones also during the HTL of biomass. Thus, the EEQ of four different effluents in Figure 5.14 (C) decreased from 154 to 11.6µg/L with increasing temperatures, which displayed an opposite trend from the EC_{50}. The most potent sample, which was produced at a reaction temperature of 250°C, had the lowest EC_{50} and had the highest EEQ based on Figure 5.14 (B) and (C). The other samples demonstrated a similar trend. Therefore, utilizing a higher temperature for the HTL process was more effective in mitigating the estrogenic potency as well as removing hormone compounds from biomass feedstocks.

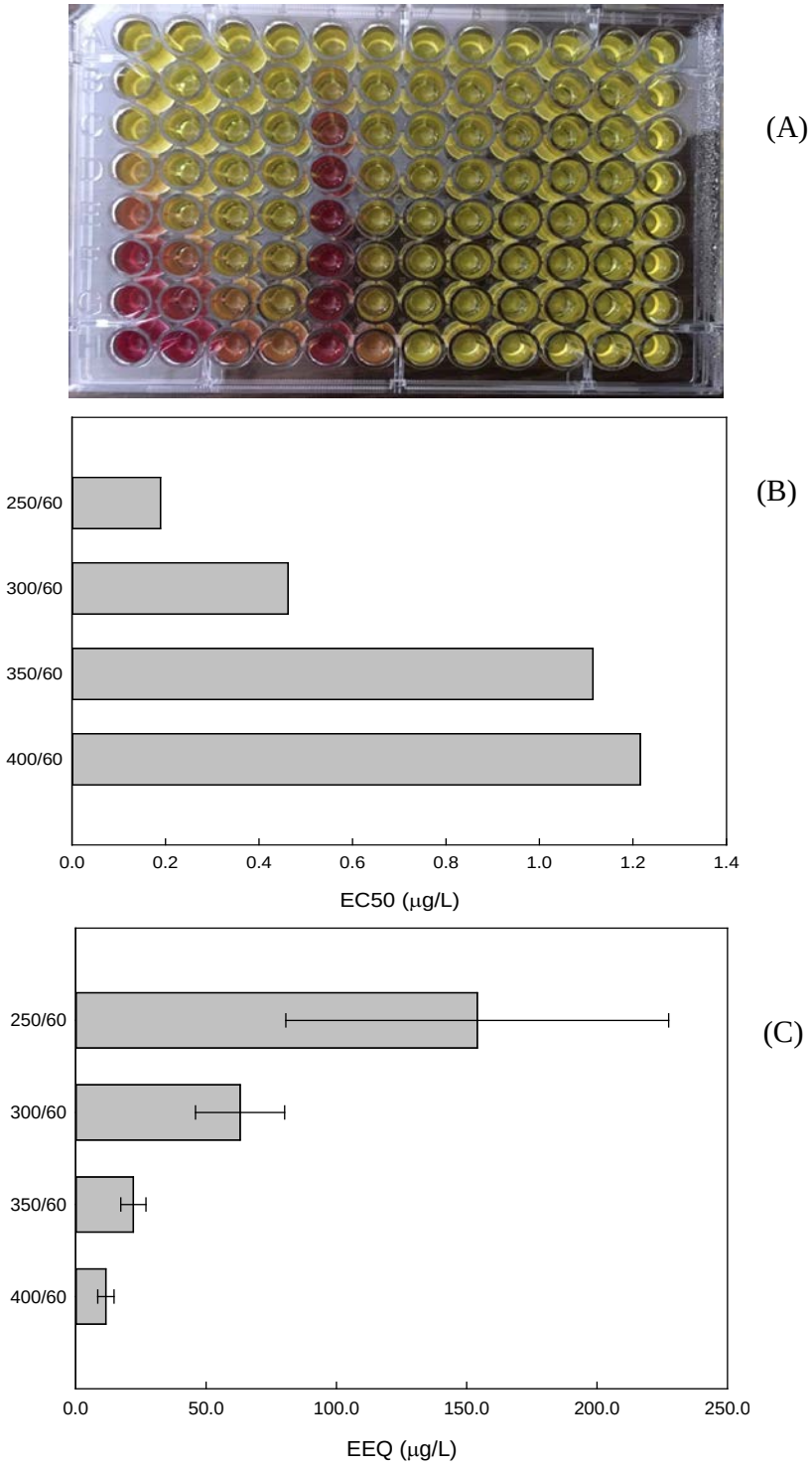


Figure 5.14(A) Plate showing the response of the yeast screen to environmental estrogens in samples (B) EC50 for agonistic activities and (C) EEQ of the HTL-WW with different reaction temperature. The error bars indicate the standard error of the mean

5.3.5.2 ESTROGENIC ACTIVITY IN CHG-WW

The Xenoscreen YES assay was used to investigate the effects of CHG operating parameters on removing endocrine disrupting effects. The methods in Chapter 3 outlined all procedures concerning sample extraction and preparation. The effects of operating conditions in the CHG of biomass on reducing estrogenic CECs and its activities would be evaluated by the residual concentrations of hormones and estrogenic activity assay.

Figure 5.15 (B) showed the agonistic response of the yeast to CHG-WW with various reaction temperatures. The EC_{50} values of temperature between 350°C and 600°C ranged from 0.1 to 1.7µg/L. According to the EC_{50} values, the reaction temperature 400°C had the lowest EC_{50} and was the most potent among the tested samples, and other the EC_{50} values was gradually increased. This demonstrates that higher CHG of biomass reaction temperatures reduces the estrogenic activity of CHG-WW, which was similar to the reduction trend of estrogenic hormones during biomass CHG as well. Thus, EEQ of six different CHG-WW in Figure 5.15 (C) decreased from 303.5 to 17.4 µg/L with increasing temperatures, which demonstrated a backwards trend from EC_{50}. The sample with the most potency, obtained at a reaction temperature of 400°C, had the highest EEQ but the lowest EC_{50} according to Figure 5.15 (B) and (C). A similar trend could be seen from the other samples. Thus, removing estrogenic activities and hormone compounds from biomass feedstock was more effective with CHG at higher temperatures. These results are supported by Routledge and Sumpter, (1996)'s work, which demonstrated that the YES assay indicated a high degree of specificity for estrogenic compounds in contrast to the YAS assay.

A dose-dependent elevation in synthesis of β-galactosidase in the yeast was produced by the CHG-WW obtained at different temperatures of CHG tests, but all doses were less potent than

E2 (fold less potency given in parentheses). This demonstrates the reducing estrogenic activity capacity by increasing temperature of biomass CHG. Estrogenic activities were reduced in the CHG-WW from higher temperature CHG, so high temperature generated CHG-WW which is less estrogenic by removing estrogenic compounds.

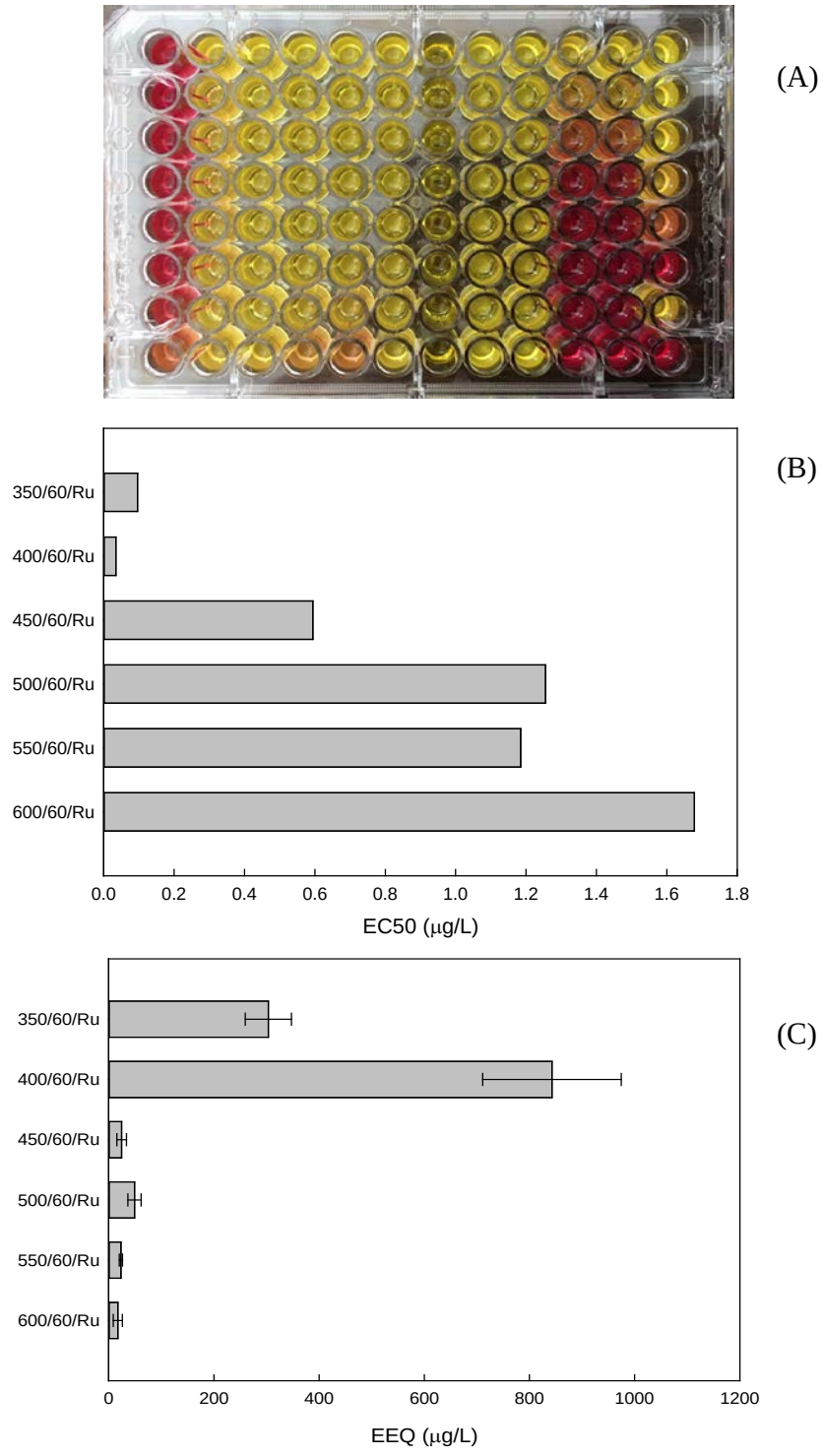


Figure 5.15 (A) Plate showing the response of the yeast screen to environmental estrogens in samples (B) EC_{50} for agonistic activities and (C) EEQ of the CHG-WW with different reaction temperatures. The error bars indicate the standard error of the mean

5.3.5.3 ESTROGENIC ACTIVITY IN SEQUENTIAL HTL/CHG-WW

As the reaction temperature increased, the EC_{50} value of HTL/CHG-WW similarly increased as demonstrated in Figure 5.16 (B). Specifically, as the reaction temperature was raised from 400°C to 600°C, the EC_{50} of HTL/CHG-WW increased from 0.15 to 0.72 μg/L. Among the tested samples, CHG at 400°C had the lowest EC_{50} and was also the most potent according to the EC_{50} values, and other EC_{50} values gradually increased in correlation to higher temperature CHGs. This indicates that estrogenic activity of HTL/CHG-WW is reduced by higher temperatures in CHG. Thus, Figure 5.16 (C) displays the EEQ of five different samples which decreased from 121.1 to 40.4 μg/L in correlation to the increase in reaction temperatures, which described a reverse trend in EC_{50}. Therefore, relieving estrogenic activities and reducing hormone compounds from biomass feedstock subjected to the HTL/CHG process was more effective at higher temperatures.

E2 was the most potent compound in comparison to the dose-dependent elevation in synthesis of β-galactosidase in the yeast produced by the CHG-WW from different temperature CHG tests. Increasing the temperature during the CHG of biomass feedstock markedly reduced estrogenic activity and removed hormone compounds (Figure 5.16). Therefore, the estrogenic potency of HTL/CHG-WW was reduced by higher temperatures, and EEQ decreased sharply at temperatures higher than 550°C during the CHG of HTL-WW, which demonstrated minimal estrogenic activity of higher temperature HTL/CHG-WW.

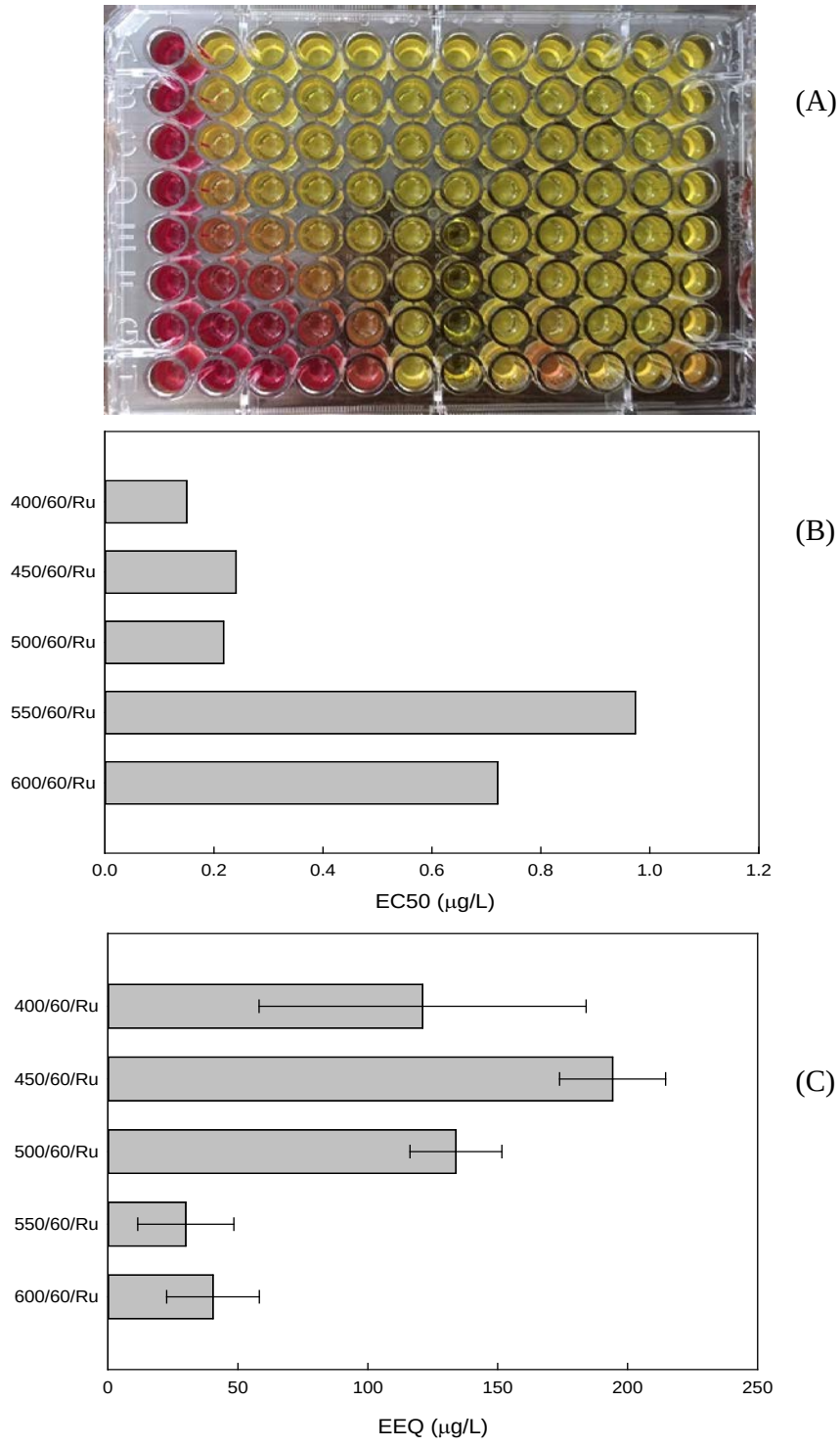


Figure 5.16 (A) Plate showing the response of the yeast screen to environmental estrogens in samples (B) EC_{50} for agonistic activities and (C) EEQ of the HTL/CHG-WW with different reaction temperature. The error bars indicate the standard error of the mean

5.3.6 ANTIBIOTIC RESISTANCE OF HTL-WW, CHG-WW, AND HTL/CHG-WW

To determine if HTL and CHG can reduce the capacity of bacterial cells to generate antibiotic resistance, antibiotic resistant bacteria (ARB) assay tests were conducted with HTL-WW and CHG-WW. Artificially spiked biomass and HTL-WW were processed by HTL and CHG, respectively, and then survival rates were measured to determine if the antibiotics in the selection phase conferred antibiotic resistance after culturing bacteria in each medium. Figure 5.17 demonstrated that the ARB Assay is sensitive and can detect the selective pressure as illustrated in negative control versus positive control data of LB and MABB effluent. In conclusion, ARB assay is sensitive to measure antibiotic resistance, and the HTL and CHG processing could eliminate the capacity of Samples to generate FF-resistant bacteria.

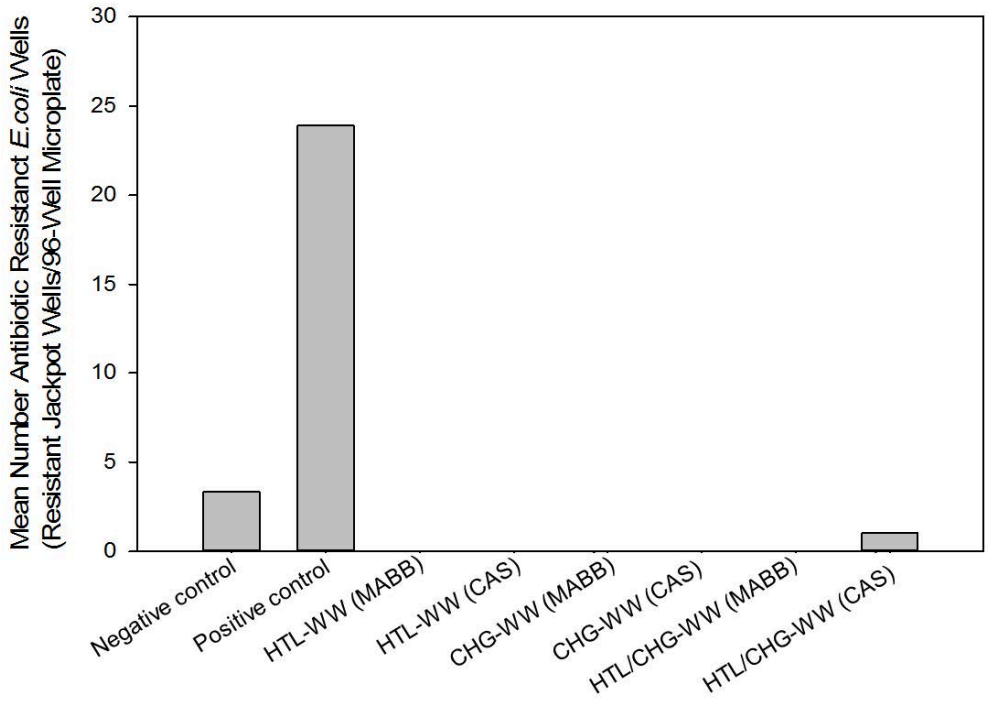


Figure 5.17 The results of the ARB fluctuation assay of samples from HTL and CHG generated low or no antibiotic resistant jackpot wells in the microplates

a. LB positive/negative: Luria - Bertani medium with/without FF spiking

b. HTL/CHG: CHG-WW of HTL-WW from MABB/CAS biomass

5.3.7 CORRELATION BETWEEN OPERATING CONDITIONS AND CECS REMOVAL

To evaluate the effects of CHG and HTL operating conditions on the removal of CECs such as estrogenic hormones and FF, a Pearson correlation analysis was conducted. This correlation analysis measures the dependence and linear correlation (dependence) of two variables X and Y. Measurements of the correlation coefficient vary from -1 to 1. The relationship between X and Y is determined perfect if all data points fall on a straight line in which Y increases as X increases, corresponding to a value of 1, implying a linear equation. A value of -1, on the other hand, that all data points fall on a line in which Y decreases as X increases. A value of 0 states that there is no linear correlation between the variables. The removal of estrogenic hormones and FF

as well as the P values and correlation coefficients (r) of multiple operating conditions are displayed on Table 5.8. Pairs of variables tend to increase together if their P values are below 0.05 and correlation coefficients are positive. In contrast, one variable tends to decrease while the other increases if their P values are below 0.05 and correlation coefficients are negative. Finally, P values greater than 0.05 equate to no significant correlation or relationship.

When analyzing the correlation coefficients for the removal of estrogenic hormones and the operating conditions of HTL, some positive and significant relationship were occurred with the operating conditions of HTL and/or CHG for the removal of hormones and/or FF. For example, Table 5.8 depicts a direct positive relationship between reaction temperature and E1 removal because of the quantified values of E1 removal (r=0.99, P=0.02) during biomass HTL. Therefore, by increasing the reaction temperature, more E1 was removed in biomass HTL, with which we can infer that a significant relationship exists between E1 removal and reaction temperature. However, the removal of total hormone compounds and HTL operation parameters had insignificant relationships, determined by correlation coefficients and the P value. Figure 5.18 (A) displays the CHG operating conditions as well as each corresponding rate of estrogenic hormones removal. The removal of total hormones has a positive correlation with CHG reaction time (r=0.95, P=0.05), as indicated by Table 5.8. Also, the removal of total hormones has a significant relationship with reaction temperature (r=0.89, P=0.04), which can be inferred through statistical analysis. As a result, higher total hormones removal was probable with higher reaction temperatures and longer reaction times in CHG tests. Thus, a significant relationship was established between reaction temperature and E2, and also between reaction time and E2, E3, EE2 (Table 5.8) for biomass CHG. When different amounts of a Ru catalyst were applied in CHG, only EE2 demonstrated a positive relationship (r=0.97, P=0.01).

Reaction temperature was the only operating condition demonstrating a direct positive relationship with FF removal (r=0.99, P=0.01) during HTL as according to Table 5.8, which indicated increasing the reaction temperature of HTL removes more FF. But, reaction time and FF removal had no significant relationship. Thus, insignificant relationships were shown based on the correlation coefficient (r) and P value between HTL operation parameters and FF-BP concentration in HTL-WW, which stated that the distribution of FF-BP to HTL-WW was unaffected by the reaction temperature and time during HTL.

Figure 5.18 (B) showed the CHG operating conditions and the corresponding FF removal. FF removal and reaction temperature of CHG had a positive correlation indicated by Table 5.8, and a significant relationship between the two variables was demonstrated by statistical analysis (r=0.86, P=0.003). As a result, higher FF removal was possible by higher reaction temperature in CHG tests. Thus, there was insignificant relationship between operating parameters in CHG and FF-BP distribution. During the sequential HTL/CHG tests, the reaction temperature was the only variable that showed a positive relationship with the removal of total hormones (r=0.99, P=0.01). Other variables such as reaction time and catalyst didn't affect any of the results of estrogenic hormones during the CHG of HTL-WW.

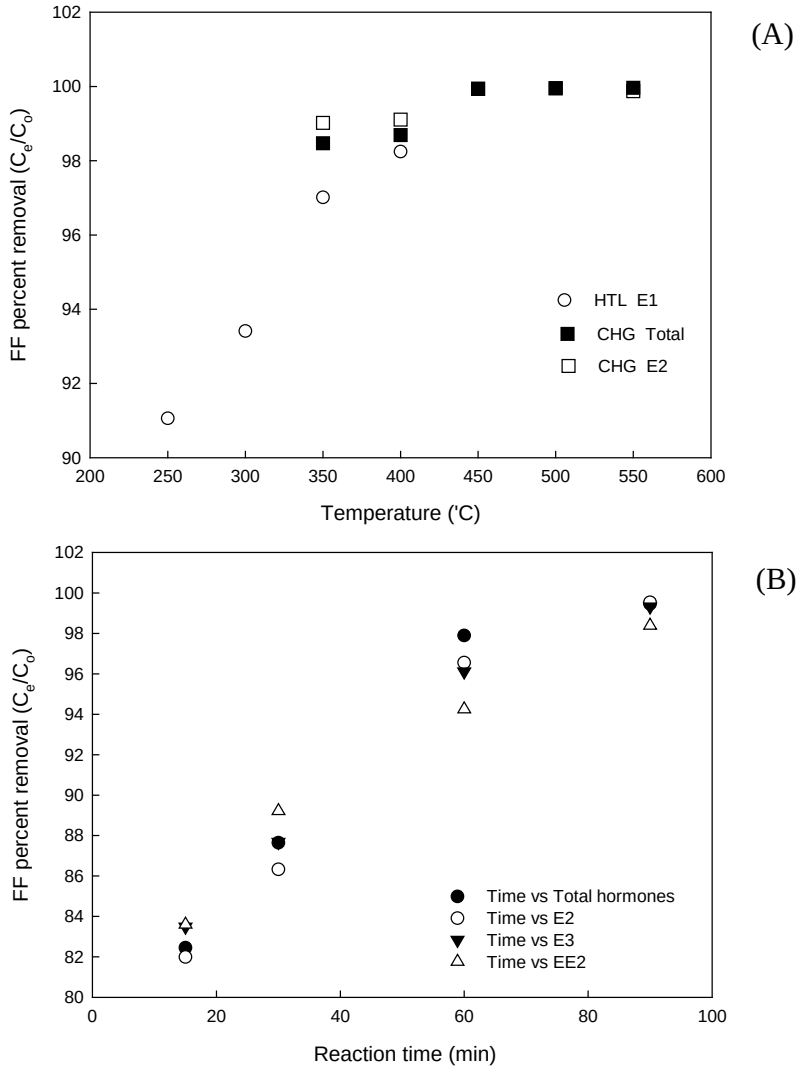


Figure 5.18 (A) Correlation between reaction temperature of hydrothermal processes and removal of estrogenic hormones (B) Correlation between reaction time of hydrothermal processes and removal of estrogenic hormones

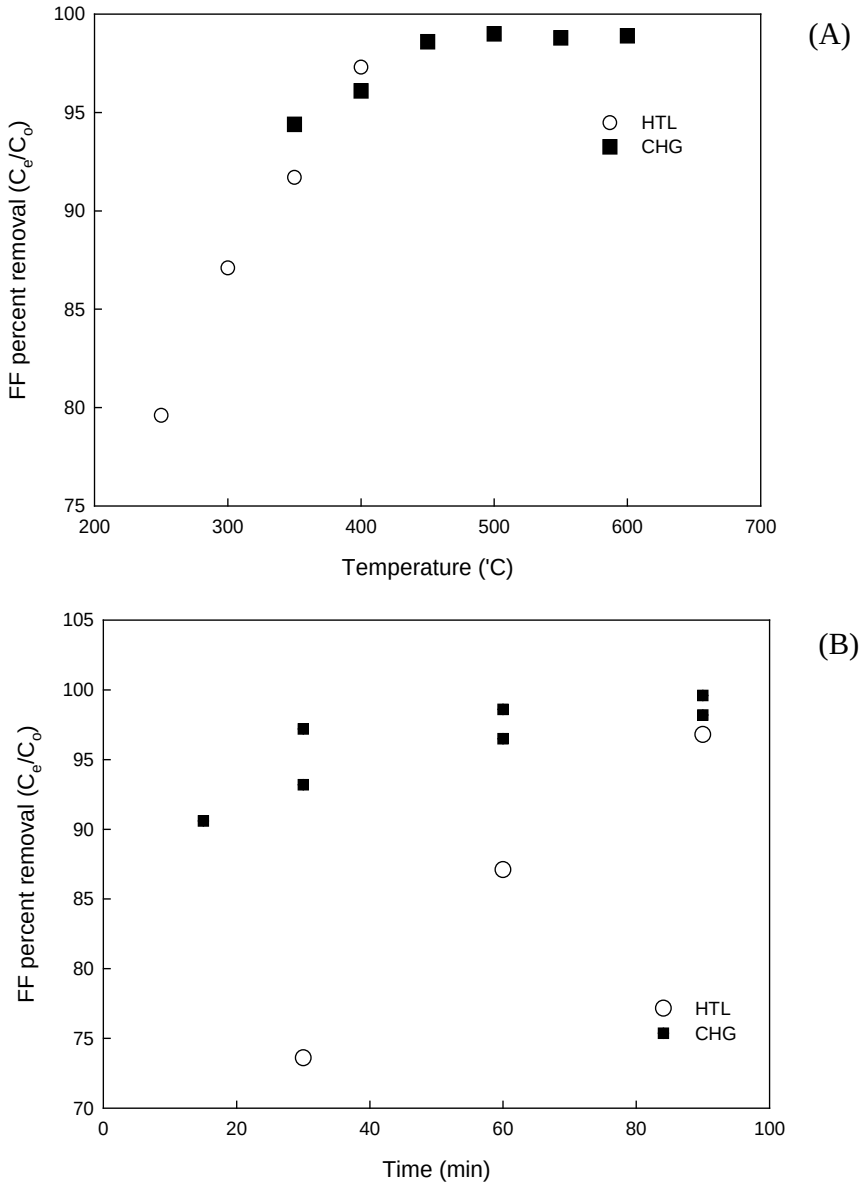


Figure 5.19 (A) Correlation between reaction temperature of hydrothermal processes and FF removal (B) Correlation between reaction time of hydrothermal processes and FF removal

Table 5.8 Correlation between operating parameters of hydrothermal processes and removal of estrogenic hormones.

Operating parameters		Total		E1		E2		E3		EE2	
		Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value
HTL	Temperature (°C)	0.153	0.847	0.985	0.015	0.226	0.774	0.936	0.064	0.677	0.323
CHG	Temperature (°C)	0.891	0.043	0.422	0.479	0.854	0.066	0.433	0.466	0.770	0.128
	Reaction time (minute)	0.954	0.046	0.911	0.089	0.974	0.026	0.980	0.020	0.979	0.021
	Catalyst amount (g)	0.662	0.223	0.453	0.443	0.609	0.276	0.626	0.259	0.868	0.056

Table 5.9 Correlation between operating parameters of hydrothermal processes, FF removal, and FF breakdown products in aqueous phase.

	Operating parameters	FF removal		FB: MPS		FB: 4-MSB		FB: 4-MSAP		FB total	
		Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value
HTL	Temperature (°C)	0.995	0.005	0.775	0.225	0.258	0.742	0.258	0.742	0.318	0.682
	Reaction time (minute)	0.996	0.060	-0.895	0.295	-0.849	0.355	-0.903	0.282	-0.896	0.293
CHG	Temperature (°C)	0.860	0.028	-0.536	0.273	-0.035	0.947	-0.347	0.500	-0.393	0.441
	Reaction time (minute)	0.834	0.166	0.981	0.019	0.153	0.847	0.304	0.696	0.416	0.584
	Catalyst amount (g)	0.103	0.869	0.089	0.887	-0.381	0.527	-0.395	0.510	-0.393	0.513
HTL/ CHG	Temperature (°C)	0.986	0.014	-0.775	0.225	-0.258	0.742	-0.258	0.742	-0.477	0.523
	Reaction time (minute)	0.983	0.116	NA	0.833	0.000	NA	0.000	NA	0.000	NA

5.4 CONCLUSIONS

This study investigated the effects of thermochemical bioenergy production processes including the HTL and CHG on the fate of bioactive CECs. The removals of E1, E2, E3, EE2, and total hormones were strongly affected by the operating conditions of HTL and CHG for biomass conversion. After running the HTL at 300°C/60minutes, the residual concentrations of total hormones ranged from 8 to 20 µg/L in the HTL-WW. The optimal HTL condition to simultaneously provide the highest oil yield and hormones removal was 300°C/60minutes, which demonstrates a 40% oil yield (dry basis) and 95.4% removal of total hormones in average. The removal of FF during HTL tests enhanced from 79.6% to 97.3% as the reaction temperature was increased from 250 to 400°C, and FF concentrations were below the detection limit of GC/MS at 400°C, which means the reaction temperatures in biomass HTL are significantly related to the removal of FF ($r=0.99$, $P=0.04$). Thus, the optimal condition for HTL processes to simultaneously remove FF and FF-BP in HTL-WW was at 400°C/60minutes, which resulted in FF percent removals of 97.3% and a total distribution of FF-BP to HTL-WW of 34.1%. However, Table 5.8 demonstrated an insignificant difference of percent distribution of FF-BP during HTL under different reaction temperatures ($P=0.68$) and times ($P=0.29$).

In CHG tests, the removal of estrogenic hormones, except for EE2 and total estrogenic compounds, was not sensitive to the temperature changes above a critical point (temperature: 374°C, pressure: 22.1 MPa) because the percent removal of the E1, E2 and E3 hormones ranged from 99.87 to 99.99% at temperatures higher than 450 °C. The percent removal of estrogenic hormones was proportional to increased reaction time, and 99.7% of total hormones were removed after 90 minutes of biomass CHG. When five different combinations of catalyst were investigated, Ru/NaOH was the most effective catalyst in breaking down estrogenic hormones during the CHG

process out of 5 different catalyst combinations. The performance of NaOH as an alkali catalyst for hormone removal was promising, but it did not enhance removal when combined with other metal catalysts such as Ru and Ra-Ni. When increased amounts of the Ru catalyst was applied in the CHG, more estrogenic hormones were removed, excluding EE2. Furthermore, the biomass and catalyst ratio which showed the highest percent removal (total hormones, 99.1%) was 9:1 during CHG tests at 350˚C/60minutes. The baseline concentration of total hormones in HTL-WW for sequential HTL/CHG tests was 8 to 20 µg/L, and the percent removal with Ru, Ra-Ni, and NiO catalyst in sequential HTL/CHG treatment was 99.8, 99.6, and 99.9%, respectively. The hormones removal in sequential HTL/CHG was not sensitive to the type of catalyst because biomass HTL followed by CHG was already strong enough to remove most of the hormones in the biomass.

The FF removal rates were gradually enhanced with the increasing temperature from 350°C to 600°C in biomass CHG, and then they plateaued to between 98.6% and 99.0% at temperatures higher than 450°C because the highest removal of FF was observed at CHG temperatures of 450 °C or higher, following the general hormones removal trend. Thus, there was a significant relationship between the reaction temperature and the removal of FF ($r=0.86$, $P=0.03$) in CHG. The percent distribution of each FF-BP such as MPS, 4-MSB, 4-MSAP, and their combined total ranged from 0.1 to 1.9%, 0.9 to 1.8%, 4.4 to 11.5%, and 5.4 to 14.2% based on the original amount of spiked FF. Thus, these results did not demonstrate any trends or relationships with the increase of reaction temperatures ($r=-0.39$, $P=0.44$) in CHG tests. Ra-Ni/NaOH demonstrated the highest percent removal of FF among the 5 different combinations of catalysts. More than 94.1% of FF can be removed through the biomass CHG, and NaOH catalyst showed the lowest FF removal compared to other catalysts and increased the distribution of FF-BP to CHG-WW.

As the catalyst dose in CHG was increased from 0.1 to 4g per 10g biomass, the percent removal of FF during CHG tests were increased from 88.9 to 98.7% and stabilized to around 98.6%. However, the percent distribution of FF-BP to CHG-WW gradually decreased with increasing amounts of the catalyst from 39.7 to 2.6%, which meant most of the FF-BP was removed from the aqueous phase with higher dose of catalyst. According to a statistical analysis on the correlation between the catalyst amount and the removal of FF and FF-BP Table 5.8, the FF removal from Biomass ($r=0.81$, $P=0.1$) and FF-BP removal from CHG-WW ($r=-0.77$, $P=0.12$) were positively related to the catalyst quantity. As the reaction time increased to 30 minutes or more, the removal of FF from biomass plateaued to 98.4% on average. Thus, the distribution of FF-BP in CHG-WW decreased from 14.2% to 7% as the reaction temperature increased from 350°C to 550°C under CHG conditions of 60minutes/Ru catalyst.

When discussing the correlation between the operating parameters of hydrothermal processes and the removal of hormones, reaction temperature and time were positively related to the removal of hormones with the biomass CHG, and a direct positive relationship was seen between the reaction temperature and E1 removal during the biomass HTL. For biomass CHG, the removal of total hormones has a positive correlation with CHG reaction time, since the removal of total hormones has increased with the higher reaction temperature ($r=0.89$, $P=0.04$) or longer reaction time ($r=0.95$, $P=0.05$) in CHG. As a result, higher total hormones removal is probable with higher reaction temperatures and longer reaction time in CHG tests.

For the relationship between FF or FF-BP and hydrothermal processes, reaction temperature was the only operating parameter which demonstrated a direct positive relationship with FF removal ($r=0.99$, $P=0.01$). Thus, the reaction temperature and time did not affect the distribution of FF-BP in HTL-WW. A statistical analysis demonstrated a significant relationship

between the FF removal and reaction temperature in CHG (r=0.86, P=0.03), which meant that higher CHG reaction temperatures would also correspond to higher FF removal.

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CHAPTER 6. CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF WASTEWATER FROM INTEGRATED MANURE MANAGEMENT SYSTEM

6.1 INTRODUCTION

HTL converts wet bio-solids (up to 85% water content) into biocrude oil, bio-char, a gaseous (predominantly CO_2), and an aqueous wastewater (HTL-WW) product with significant nutrient and organic content (Anastasakis & Ross. 2011; Brown et al., 2010; Duan & Savage. 2011; He et al., 2000; Valdez et al., 2012; Yu et al., 2011). The HTL process is an attractive option for bio-fuel production for several reasons. First, HTL works effectively on low-oil microorganisms. Second, biomass does not need to be dried before HTL processing. Additionally, the biocrude oil and aqueous wastewater separate naturally. Moreover, unused nutrients and CO_2 can be recycled for algal cultivation. HTL has the potential to effectively and efficiently reduce and neutralize bioactive organic contaminants in various wastewaters while producing biofuel for energy (Pham et al., 2013). Gai et al., (2015) reported the key variables affecting HTL performance are temperature, retention time, and solid ratio. This study will provide further characterization of the chemical and biological characteristics of HTL-WW as a function of HTL operating temperature (250-400°C) and retention time (30-60 minutes).

Another promising method which both protects the environment and produces usable energy is CHG, which removes estrogenic compounds and FF from biomass and produce biogas. CHG processing conditions are 250-360°C, and up to 22 Mpa (Elliott et al., 1988; Elliott et al., 1994). While a small amount of char is created, the main byproduct is a mixture of CO, CO_2, H_2, and CH_4 called syngas (Balat et al., 2009; Ro et al., 2007). CHG can be performed on wet organic

wastes such as biomass and livestock manure (Ro et al., 2007). Catalytic hydrothermal gasification can operate with various operating parameters such as temperatures, pressures, catalysts type and amount. According to Azadi and Farnood, (2011), nickel and ruthenium were found to be good catalysts for CHG. Thus, high-efficiency carbon gasification can be performed using ruthenium as a catalyst (Brown et al., 2010). In the previous studies, iron, cobalt, ruthenium, and nickel were tested as catalysts in CHG tests. Besides facilitating the conversion process, their other benefits include improving the quality of the byproducts and reducing the tar (Balat et al., 2009). Another study showed that potassium was an effective catalyst in corn starch but not in clover grass or corn silage (D'Jesus et al., 2005). An important goal would be to identify effective catalysts for enhancing the quality of the aqueous and improve the chemical and biological characteristics of CHG-WW products by removing CECs and toxic compounds.

Additional research was conducted on the combination of HTL and CHG process to characterize the aqueous phase from each hydrothermal process. After HTL and CHG tests with biomass, the removal of cytotoxic compounds and antibiotic resistant capacity from their wastewater was evaluated from the environmental perspectives. A further concern is heavy metals such as Pb, As, Cd, and Hg, which can be found in biomass and animal waste (Brathwaite & Rabone. 1985; Nzihou & Stanmore. 2013; Zhou et al., 2015). Therefore, the transport and distribution of heavy metals during the hydrothermal waste to energy processes by analyzing the metal compounds in the products of each test. Thus, one study showed that wastewater from swine manure that had undergone HTL still contained high levels of biological oxygen demand (BOD) (420−59,000 mg/L) and other characteristics that made it unsuitable for discharge into the environment (Appleford, 2004). Water that has been in contact with natural petroleum can present hazards to aquatic organisms (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999;

Johnsen et al., 1994; Neff et al., 2006). This implies it is reasonable to carefully test that wastewater from HTL and CHG is safe to release to the environment and to recycle for algal cultivation. There are not yet much toxicity studies on CHG-WW in the literature. However, Elliot (1992) listed 48 hazardous substances found in HTL-WW (Elliot, 1992), including phenol, toluene, benzene, 2-methylarizidine, and aziridine (Netzeva et al., 2004; Verschaeve & Kirschvolders, 1990; Weisburger et al., 1981; Yardleyjones et al., 1991; Zhao et al., 2009). Ammonia may also be toxic to aquatic organisms (Camargo & Ward, 1995; Scott & Crunkilton, 2000), and past studies have observed that HTL-WW has to be diluted at least 20-fold in order to culture algae (Jena et al., 2011b; Zhou, 2010; Zhou et al., 2011).

The specific objectives of this study were to: 1) Investigate the effect of key HTL and CHG operating parameters (reaction temperature, reaction time, catalyst type, and catalyst amount) on the acute and CHO cell cytotoxicity of HTL-WW and CHG-WW, 2) Investigate the effect of HTL or CHG operating parameters on the distribution of heavy metals, and 3) Evaluate the correlation between the overall organic concentration and toxicity of CHG-WW.

6.2 MATERIALS AND METHODS

6.2.1 CHEMICALS AND REAGENTS

Distilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade organic solvents such as n-hexane, acetone, acetonitrile, methanol, or dichloromethane were purchased from Fisher Scientific (Fair Lawn, NJ). All Chemicals and solvents in this research were purchased at a HPLC grade.

6.2.2 HYDROTHERMAL LIQUEFACTION AND CATALYTIC HYDROTHERMAL GASIFICATION OF BIOMASS

To study the effects of HTL operating parameters on the chemical and biological characteristics of HTL-WW and CHG-WW, a total of 15 different combinations of operating conditions such as various temperature parameters (250°C-600°C), four reaction times (15, 30, 60, and 90 minutes), three different catalysts (Ru, Ra-Ni, and NaOH) used for HTL and CHG tests with biomass feedstock. All the products from HTL and CHG tests were collected and prepared for the characterization of the aqueous phase and distribution of metal compounds. Afterwards, 10 g of the biomass feedstock was placed into the batch reactor and run under different operating conditions. The HTL and CHG experiments were conducted following the same method and separation of HTL and CHG products followed the procedure outlined in Chapter 5.

6.2.3 CATALYTIC HYDROTHERMAL GASIFICATION OF HTL-WW

HTL-WW was produced from the HTL tests with biomass under 300°C and 60 minutes, and HTL-WW was spiked with the stock solution of estrogenic hormones (E1, E2, and E3 at 0.5 mg/mL) and FF (1.0 mg/L) to investigate the mitigation of CECs and reuse of aqueous portion of wastewater by CHG. Based on the favorable results from the previous studies in Chapter 5, increasing temperature from 300°C to 600°C were applied to perform the preliminary tests of combined HTL and CHG operations for removing the residual organics and toxic compounds from the HTL-WW. Three different types of catalyst such as Ra-Ni, Ru, and NiO were selected to compare the performance of CHG tests on HTL-WW as well.

6.2.4 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF HTL-WW AND CHG-WW

6.2.4.1 WATER QUALITY ANALYSIS

The sCOD of all the HTL-WW, CHG-WW, and HTL/CHG-WW was analyzed to investigate the effects of various operating conditions of hydrothermal processes on the removal of dissolved organics from the aqueous phase.

6.2.4.2 HEAVY METALS

Based on the method in Chapter 3, distribution of heavy metals to aqueous phase during HTL, CHG, and HTL/CHG of biomass were explored by analyzing the heavy metals in biomass feedstock, oil phase, aqueous phase, and residue from each test under favorable conditions such as 300°C/60minutes, 400°C/60minutes/Ru, and 500°C/60minutes/Ru for HTL, CHG, and HTL/CHG, respectively.

6.2.4.3 CHO CELL MAMMALIAN CYTOTOXICITY

CHO cell mammalian cytotoxicity of outputs from HTL and CHG tests were investigated according to the method in Chapter 3. The lowest cytotoxic concentration was found at the lowest concentration of the HTL-WW sample in the concentration-response curve that induced a statistically significant reduction in cell density compared to the concurrent negative controls. Determined from data regression analysis, the LC_{50} value is the concentration of the wastewater sample that induced a cell density of 50% compared to the concurrent negative controls. The samples were not extracted using XAD resins because the aqueous products were highly concentrated for the assay, and the specific organic concentrations for CHO cell cytotoxicity tests were determined based on the sCOD results of each sample (Pham. 2013).

6.2.4.4 ACUTE TOXICITY

Microtox® from Modern Water (New Castle, DE, USA) was used to analyze the acute toxicity of aqueous products from each hydrothermal process based on the method in Chapter 3. All the wastewater samples were filter using 0.45 µm filter prior to analysis.

6.3 RESULTS AND DISCUSSIONS

6.3.1 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF HTL-WW

6.3.1.1 WATER QUALITY ANALYSIS IN HTL-WW

Figure 6.1 shows the effect of HTL temperature and retention time on soluble organics in the HTL-WW. These data provide that as the HTL reaction temperature and time were increased from 250°C to 400°C and 30 to 90 minutes, sCOD in HTL-WW decreased 57% and 51%, respectively. These results meant that the removal of sCOD had a statistically significant correlation with HTL reaction temperatures (r=-0.97, P=0.03), but the correlation between reaction time and sCOD was not statistically significant (r=-0.95, P=0.19). Figure 6.1 (A) and Table 5.6 showed that sCOD of HTL-WW was decreased with increasing reaction temperature from 250°C to 400°C, but the yield of biocrude oil was decreased higher than 300°C because bio-crude oil yield is highly dependent on the HTL reaction temperature, and would be directly proportional to increasing reaction temperatures. For example, liquefaction of sawdust at increasing temperatures reported the enhanced total oil yields (Karagöz et al., 2006). However, increasing the temperature beyond the maxima for oil yield inhibits biomass liquefaction, and formation of gases instead of biocrude oil is favored because of secondary decompositions and Bourdard gas reactions under higher temperature (Akhtar & Amin. 2011).

According to Figure 6.1 (B) and Table 5.6, increasing the reaction time generates higher biocrude oil yields and lower sCOD in the HTL-WW. These results can be supported by numerous studies which indicated that longer retention time induces the production of biocrude oil which subsequently resulted in lower residual organics in the aqueous products (Qu et al., 2003; Su et al., 2004; Xu & Lancaster. 2008; Yan et al., 1999). Thus, increasing the reaction time (30–120 minutes) increased gases yields, left no significant changes to solids residues, and finally lowered water soluble fraction yields (Jena et al., 2011a). Finally, increasing reaction temperature and time could increase the removal of dissolved organics (sCOD), but biocrude oil yield could be decreased higher than the maximum.

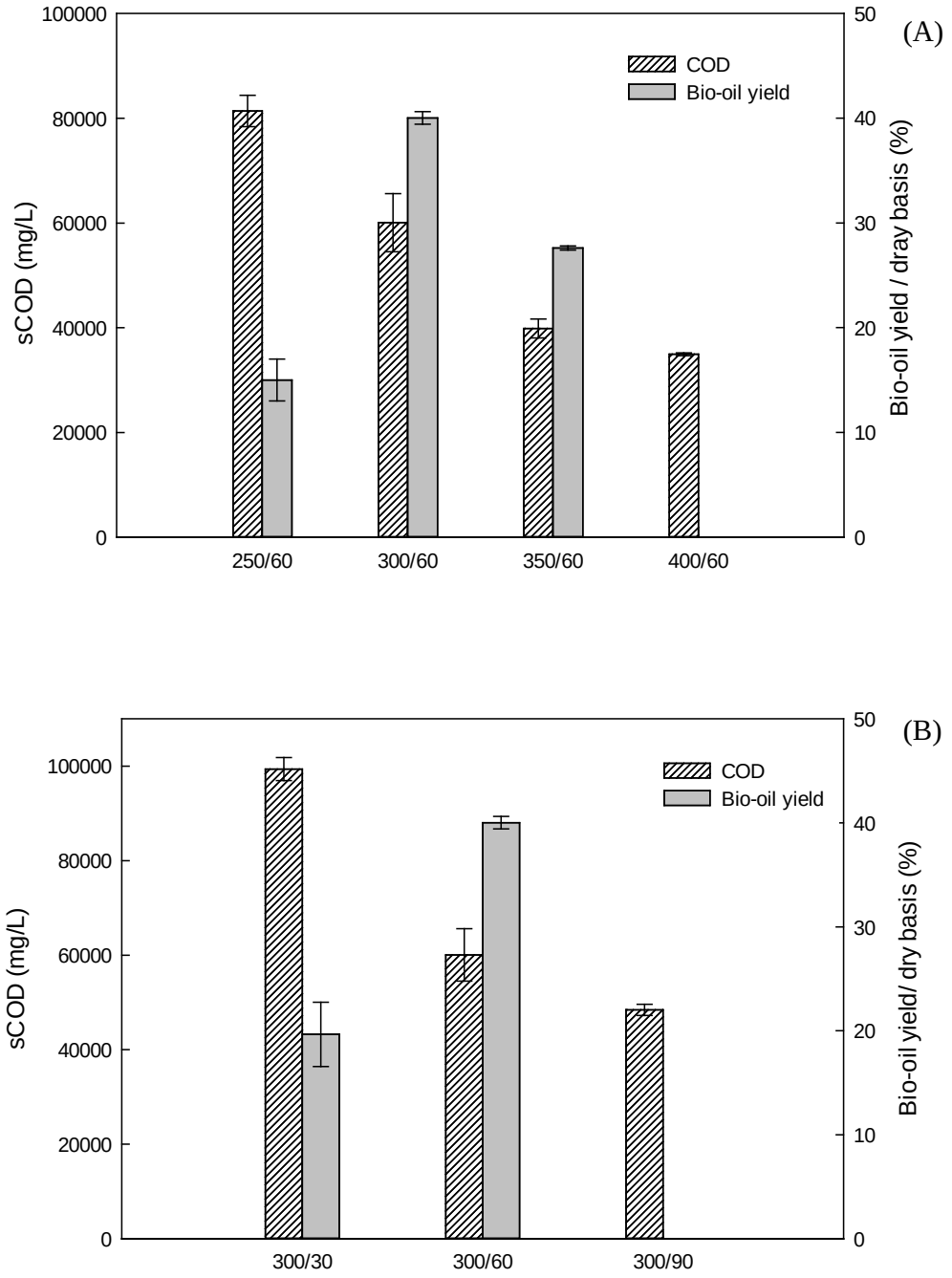


Figure 6.1 (A) Effects of reaction temperature on the sCOD in HTL-WW (B) Effects of reaction time on the sCOD in HTL-WW. The error bars indicate the standard error of the mean

6.3.1.2 HEAVY METALS IN HTL-WW

Figure 6.2 displayed the concentration of heavy metals that were measured after the HTL of biomass feedstock. Based on the concentrations of heavy metals in the biomass feedstock, the percent distribution to the HTL products such as residue, HTL-WW, and biocrude oil phase were calculated. The average percent distributions of metals from biomass to residue, HTL-WW, and biocrude oil were 69.9, 29.5, and 0.6%, respectively, which means that there was still a considerable amount of heavy metals left in the HTL-WW. In addition, the concentrations of As and Cu in the HTL-WW were 0.2 and 37.3 mg/L, which were higher than the recommended maximum concentration for irrigation and livestock drinking water (As and Cu: 0.1 and 0.2 mg/L) (Ayers et al., 1985; EPA. 1974). This could pose to be a large obstacle in the viability of the reuse of HTL-WW as a potential water source, and the fate and transport of As and Cu in the integrated manure management system has to be characterized because As and Cu could produce toxicological effects on both human health and environmental safety (Brathwaite & Rabone. 1985; Zhou et al., 2015).

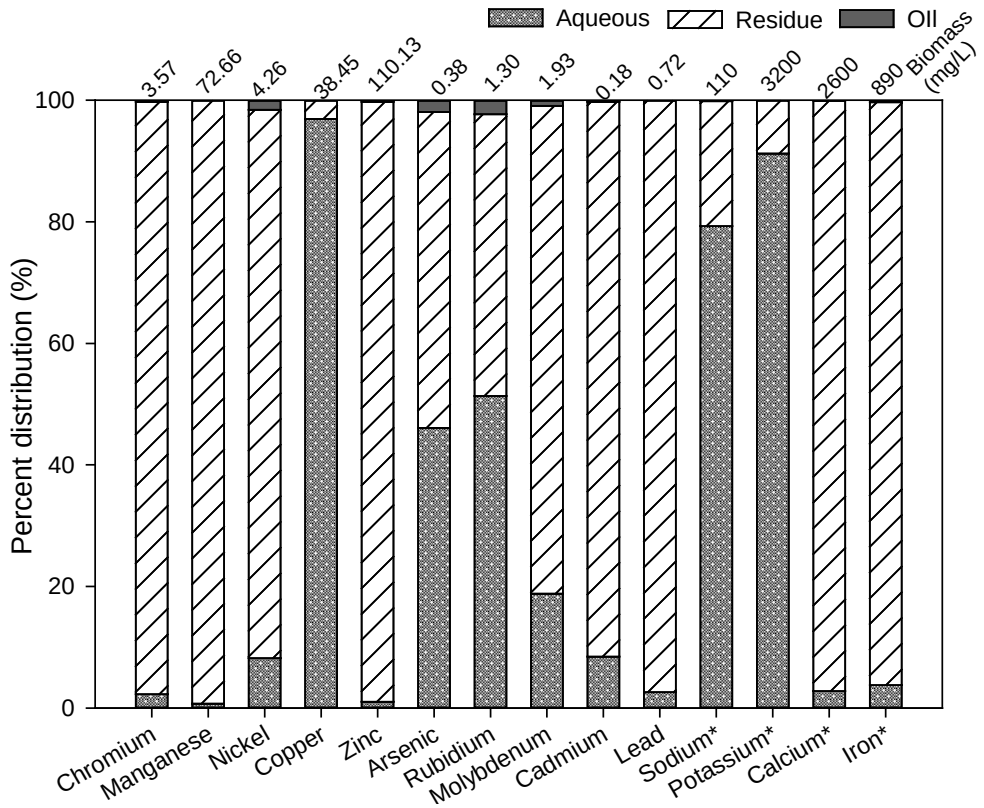


Figure 6.2 Effects of different reaction time on the removal of E1, E2, E3 and EE2 in HTL tests (n⩾2)

6.3.1.3 CHO CELL CYTOTOXICITY IN HTL-WW

The cytotoxicity of the HTL-WW at different temperatures (250˚C - 400˚C) with a fixed reaction time of 60 minutes was investigated using the CHO cell assay and is presented in Figure 6.3. These results support the following important observations. First, each HTL-WW sample induced concentration dependent cytotoxicity in mammalian cells. Second, the descending cytotoxicity potency was 400˚C > 350˚C > 250˚C > 300˚C, but the relationship between cytotoxicity and temperature was not convincing (r=0.80, P=0.19). Finally, these results confirmed that the control of the HTL process condition can be used to reduce the toxicity of the HTL aqueous product.

According Pham, (2013), increasing temperature could enhance oil yield, which subsequently resulted in the removal of sCOD from HTL-WW. Because the dissolved organics produce in the HTL-WW produce cytotoxicity, increasing temperature could lower the cytotoxicity. However, Figure 6.3 showed the increasing cytotoxicity with higher temperature, which is not identical with Pham's results. But, increased amount of oil and soluble toxic compounds could affect the cytotoxicity of HTL-WW because oil and water contacted with oil typically showed serious toxicity to aquatic species (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999; Johnsen et al., 1994; Neff et al., 2006).

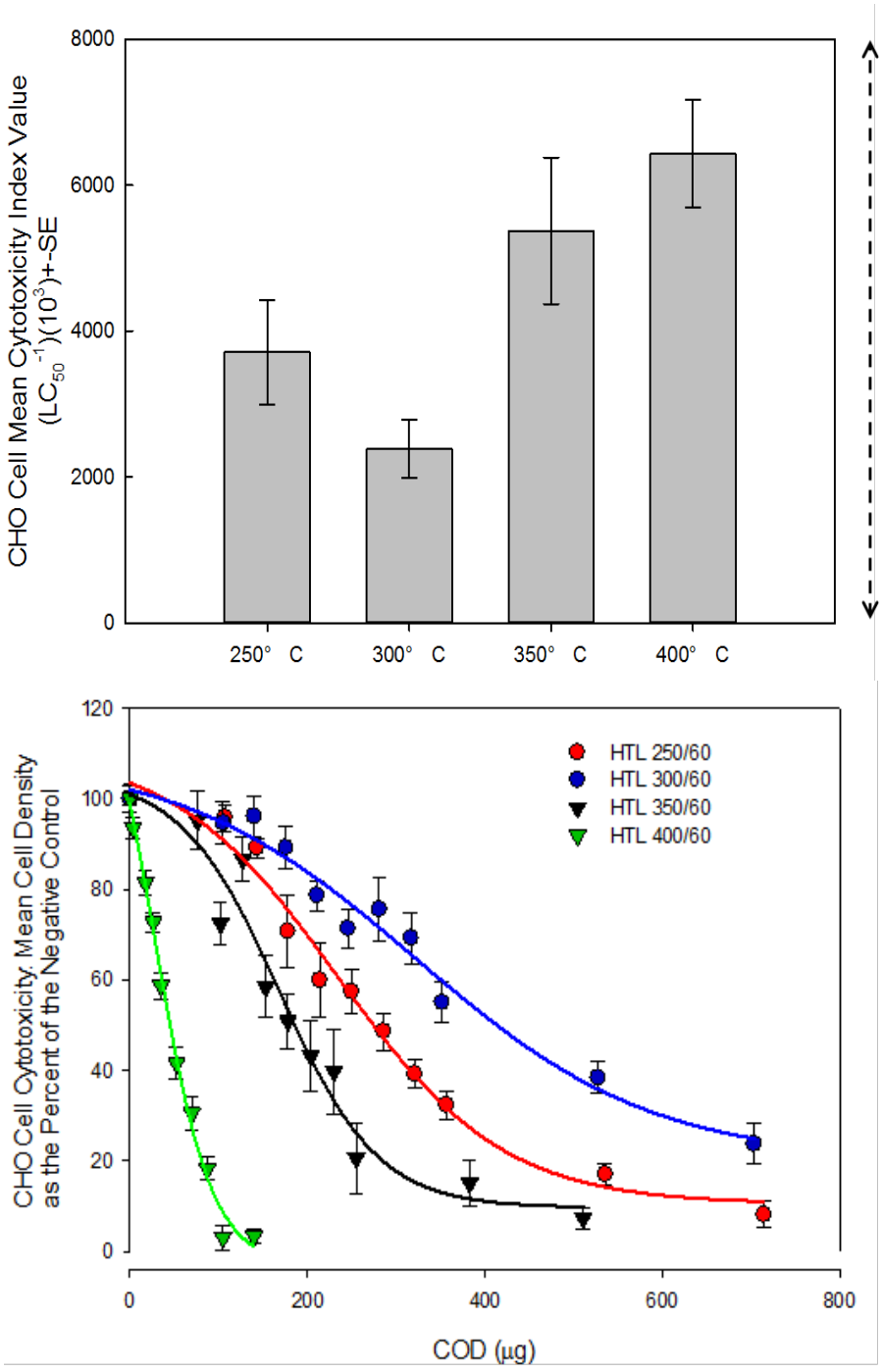


Figure 6.3 (A) CHO cell cytotoxicity index values for each HTL-WW (B) A comparison of cytotoxicity concentration response curves from HTL-WW under different temperature. The error bars indicate the standard error of the mean

6.3.1.4 ACUTE TOXICITY IN HTL-WW

The acute toxicity of the HTL-WW at different temperatures (250˚C - 400˚C) with 60 minutes of reaction time was investigated using the Microtox® assay and was presented in Figure 6.4 (A). These results supported the following important observations. First, each HTL-WW sample induced concentration dependent acute toxicity. Second, the descending microtoxic potency was 400˚C > 250˚C > 350˚C > 300˚C. Thus, these results confirmed that the control of the HTL process condition can affect the acute toxicity of the HTL aqueous product, but there was no compelling relationship between the temperature and acute toxicity (r=0.95, P=0.2), demonstrating the same trend line as cytotoxicity of HTL with increasing reaction times in Figure 6.4 (B). As stated about cytotoxicity, the increased reaction temperature could produce more toxic wastewater by contacting oil under higher temperature (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999; Johnsen et al., 1994; Neff et al., 2006).

The effects of the reaction time on acute toxicity were investigated since reaction time is critical parameter for HTL that can affect the oil yield and removal of dissolved organics (Lu et al., 2012; Susanti et al., 2011). Different reaction times of HTL from 30 to 90 minutes were tested at 300˚C for the biomass HTL, and the acute toxicity increased with the increasing reaction times. Thus, the relationship between acute toxicity and increasing reaction times in HTL were positive but not significant (r=0.95, P=0.2).

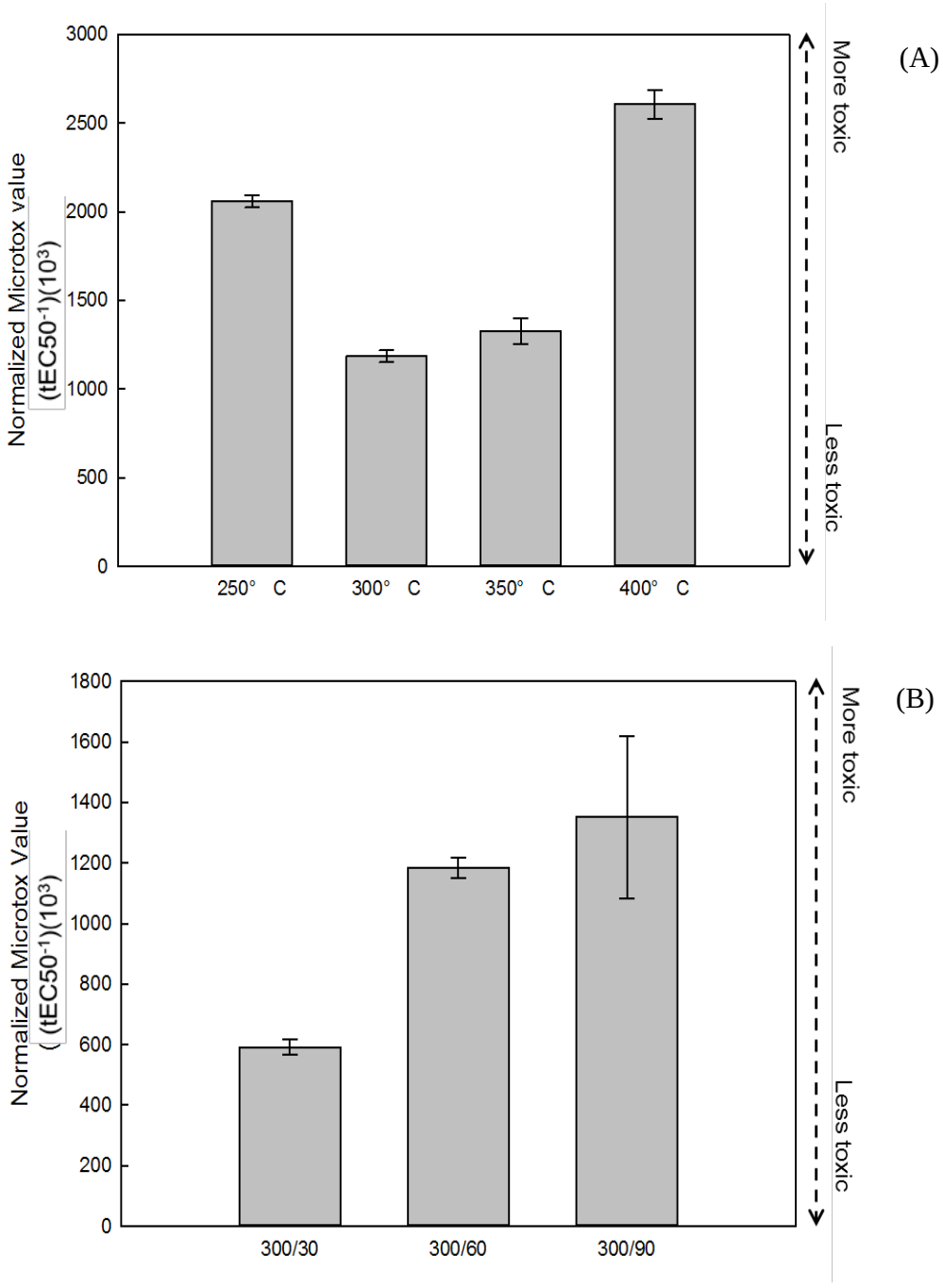


Figure 6.4 (A) Normalized acute toxicity of HTL-WW with different reaction temperature and (B) with different reaction time. The error bars indicate the standard error of the mean

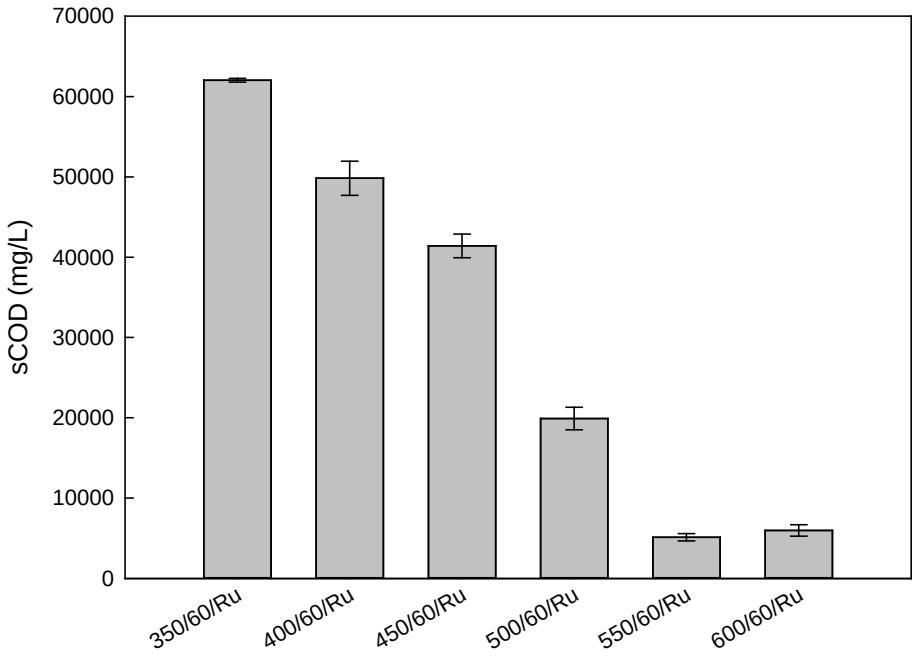
6.3.2 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF CHG-WW

6.3.2.1 WATER QUALITY ANALYSIS

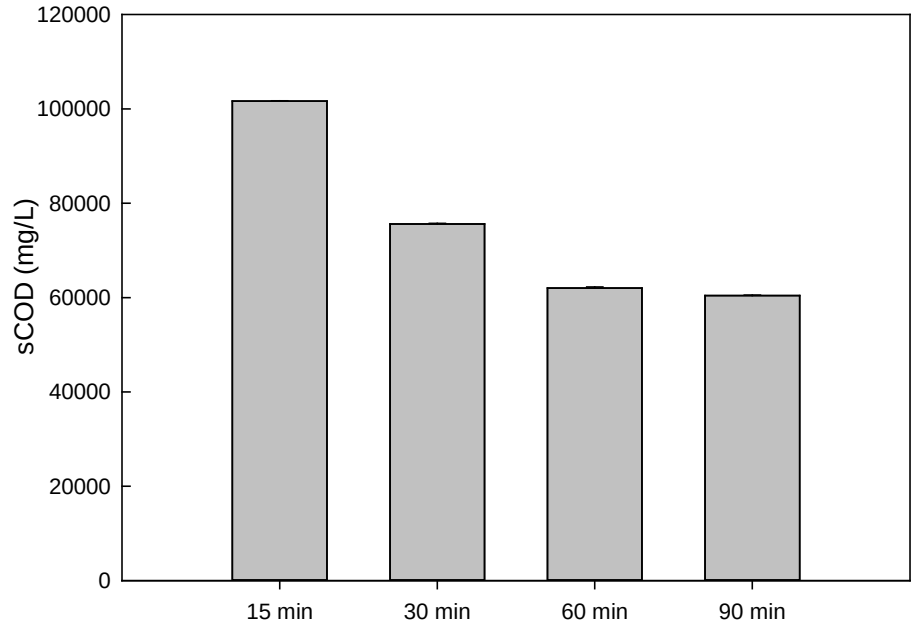
A) EFFECTS OF CHG REACTION TEMPERATURE AND TIME ON SCOD

In CHG tests, increasing reaction temperatures from 350°C to 600°C resulted in decreasing sCOD concentrations from 62,028 to 5,978 mg/L. In addition, dissolved organics were shown to gradually decrease with a significant and negative relationship (r=-0.98, P=0.001) with increasing temperatures as shown in Figure 6.5 (A). These results indicated that the reaction temperature of CHG had a major effect on the chemical and biological characteristics of the CHG-WW, which will be shown in the next section. In addition, the dissolved organics in the CHG-WW were significantly decreased under reaction conditions of 500 °C or higher with a reaction time of 60 minutes, which shows the same trend with the cytotoxicity of CHG-WW. sCOD of CHG-WW could be a good index to expect the cytotoxic impact of CHG-WW and the yield of gas production during biomass CHG.

Figure 6.5 (B) shows that increasing reaction times from 15 to 90 minutes also resulted in lower sCOD concentrations in the CHG-WW. In addition, dissolved organics were shown to gradually decrease from 15 to 60 minutes and plateaued until 90 minutes as shown in Figure 6.5 (B), but the statistical relationship was negative and not significant (r=-0.87, P=0.13). These results indicated that the reaction time contributed to lower the sCOD in CHG-WW and could have some effect on the toxicity of CHG-WW, even though there was no statistical moderate relationship between sCOD and reaction time.



(A)



(B)

Figure 6.5 (A) Effects of reaction temperature on the sCOD in CHG-WW (n ⩾ 4) (B) Effects of reaction time on the sCOD in CHG-WW under 350°C/Ru/10g biomass (n >2). The error bars indicate the standard error of the mean

B) EFFECTS OF CATALYST TYPE AND AMOUNT ON SCOD

Ru catalyst had the lowest sCOD in the CHG-WW among the 5 different combinations of metal and alkali catalysts as presented Figure 6.6 (A). The sCOD concentration in CHG-WW ranged from 24,888 to 34,243 mg/L, and the performances of the 5 different catalysts for the sCOD removal followed this order: Ru> Ru/NaOH> Ra-Ni> NaOH> Ra-Ni/NaOH. These results prove that Ru was the effective to remove dissolved organics for better energy production, and the performance of NaOH as an alkali catalyst to remove estrogenic hormones was promising, but it could not contribute to enhancing the removal of sCOD with the combination of Ru.

When the amount of CHG catalyst was increased from 0.1g to 2g per 10g biomass, sCOD in CHG-WW was not decreased effectively, but the sCOD was finally lowered from 60,000 to 48,000 mg/L, which means that an additional 20% of sCOD was decreased after adding 2g of the Ru catalyst to 10g biomass in Figure 6.6 (B). According to the results mentioned in Chapter 5, the optimal dose of the catalyst for the lowest sCOD has yet to be found through these tests due to the high capacity of water absorbance of the Ru catalyst and its price ($ 21.9/g Ru, Sigma Aldrich). The results from experiments allowed us to conclude that the sCOD levels in CHG-WW were not very sensitive to the amount of catalyst in the biomass CHG ($r=-0.83$, $P=0.17$), but Ru was better to lower the sCOD level than other catalyst.

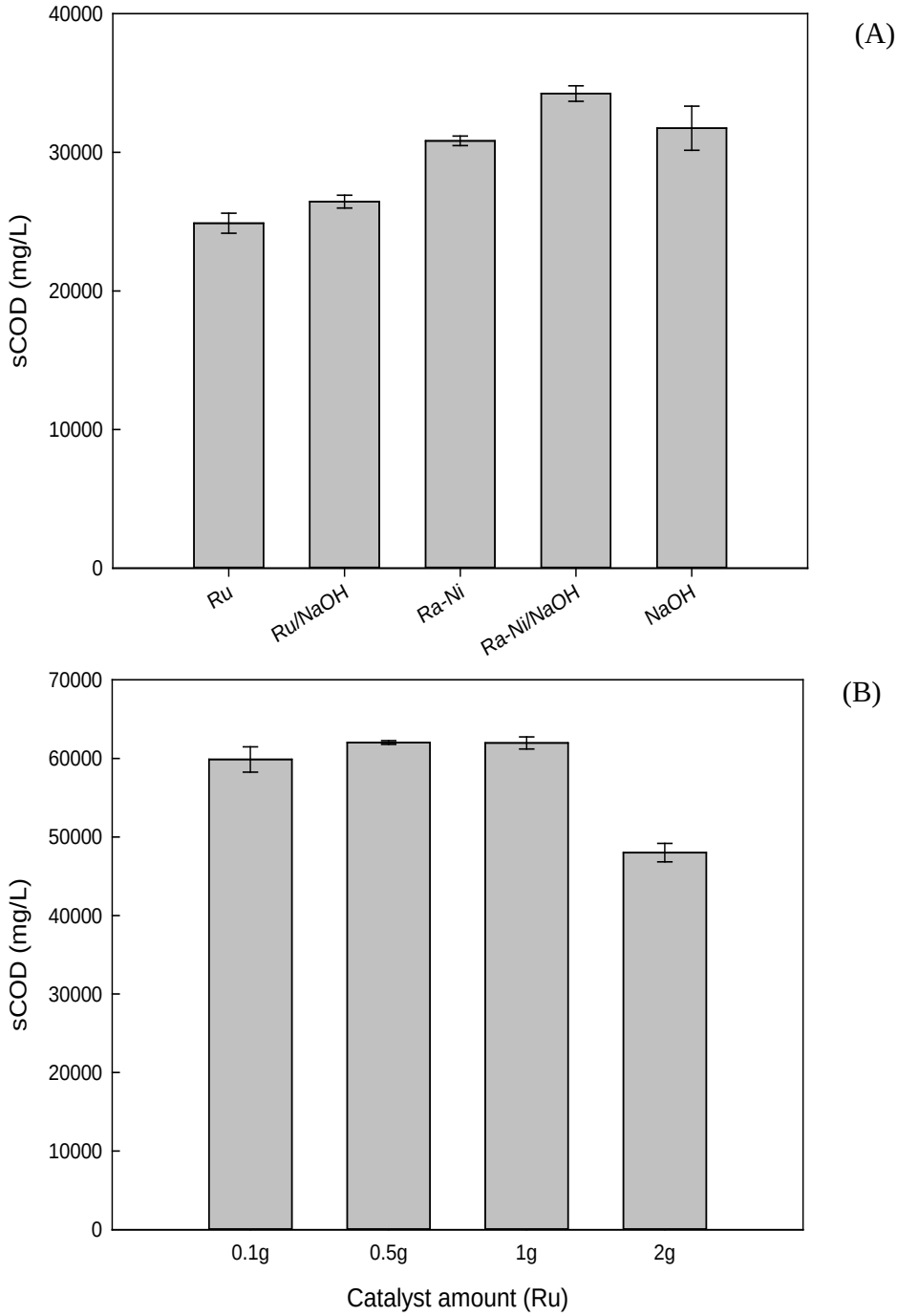


Figure 6.6 (A) Effects of catalyst type on the sCOD in CHG-WW under 400/60/Ru of CHG (n ⩾ 3) (B) Effects of catalyst amount on the sCOD in CHG-WW under 350/60/Ru of CHG (n ⩾ 2). The error bars indicate the standard error of the mean

6.3.2.2 HEAVY METALS DISTRIBUTIONS IN CHG

Figure 6.7 described the percent distribution of heavy metals to the residue, CHG-WW, and biocrude oil after the biomass CHG, and the average distribution to CHG products followed this order: residue (79.3%) > CHG-WW (18.5%) > biocrude oil (2.1%). These results showed that that most of the heavy metals transported to the residue and a substantial amount of metals, such as Mg, Cr, Cu, Zn, K, and Fe, were left in the CHG-WW after the biomass CHG under 400°C/Ru/60minutes. Especially, the concentration of toxic heavy metals such as Zn (2 mg/L), Cd (0.02 mg/L), and Pb (0.2 mg/L) in the CHG-WW were higher than the suggested maximum concentrations for irrigation water (Zn and Cd: 2.0 and 0.01 mg/L) and livestock drinking water (Pb: 0.05 mg/L). Because these toxic heavy metals could induce adverse and toxicological effects to aquatic organism, they are required to be monitored and removed using additional treatments (Ayers et al., 1985; Gai et al., 2015).

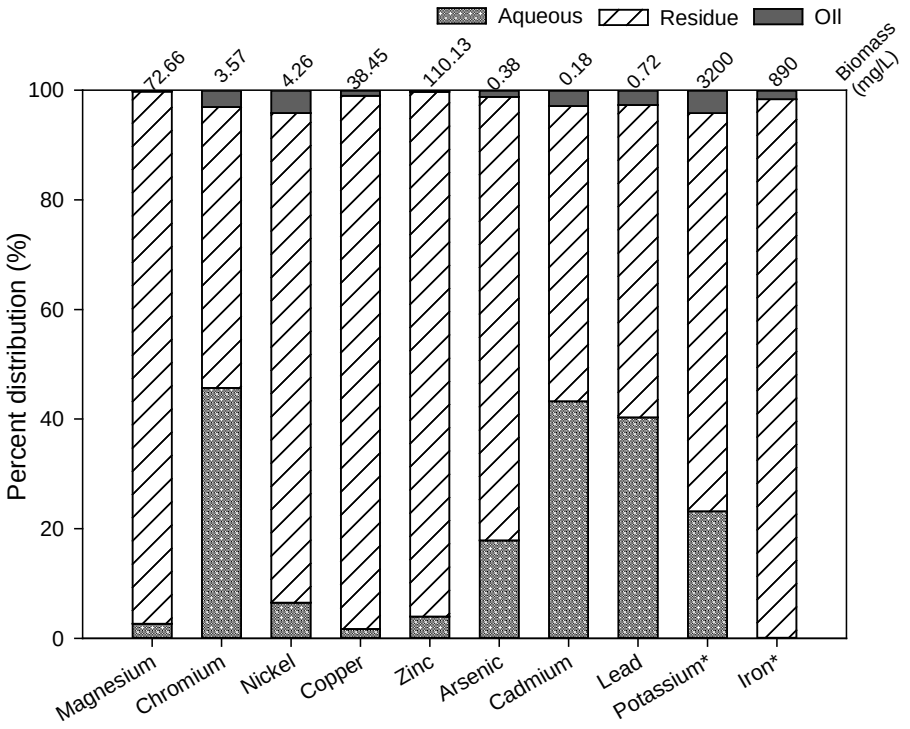
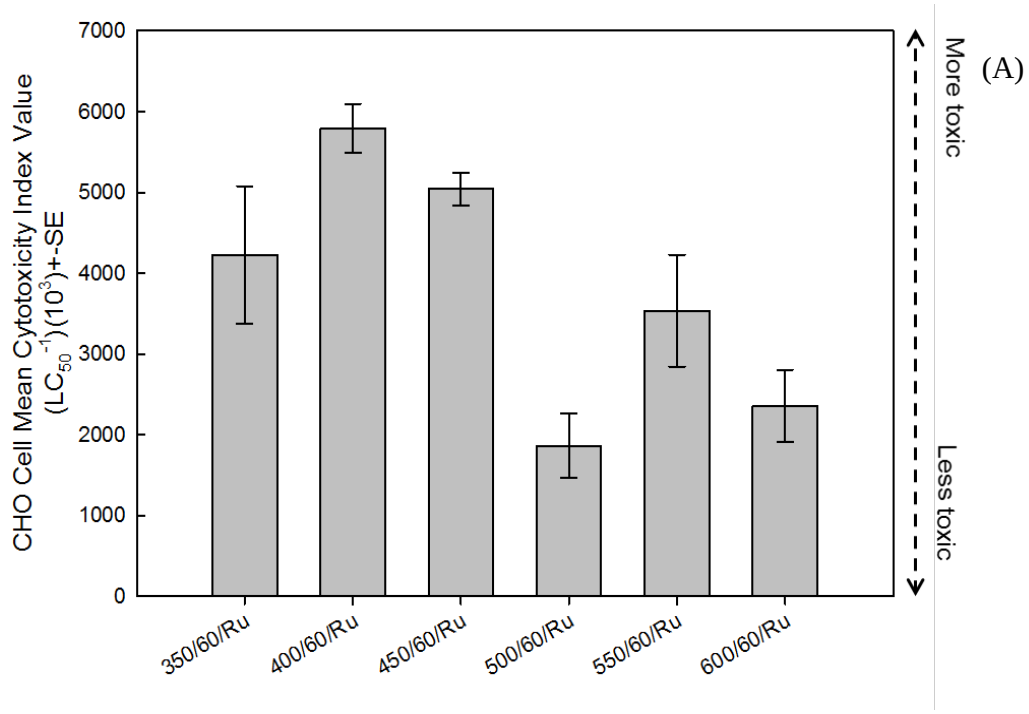


Figure 6.7 Distribution of heavy metals to CHG-WW, residue, and oil in CHG tests

6.3.2.3 CHO CELL CYTOTOXICITY IN CHG-WW

According to Figure 6.8 (B), when the reaction temperature was raised from 350°C to 400°C in biomass CHG, cytotoxicity index value of CHG-WW was increased from 4,227 to 5,792, which was the highest CTI value observed for the CHG of biomass. However, CTI values were decreased as the temperature increased from 400°C to 600ºC, which signified that toxic compounds were removed with higher temperatures under negative correlation (r=-0.68, P=0.14). Thus, the lowest CTI value in CHG-WW was observed at 500°C/60minutes/Ru, which showed the highest energy recovery (77%, dry basis) according to Table 5.6.

These results can be explained by the formation of different organic compounds with varying toxicity indexes under the different reaction temperatures, and the toxic compounds in CHG-WW could be generally removed by more effective conversion of organic compounds to syngas under higher reaction temperatures, which subsequently resulted in the higher energy recovery. Thus, it was proved that 500°C under 60minutes/Ru was the optimal condition of biomass CHG to achieve higher energy recovery and lower the cytotoxicity in the aqueous phase.



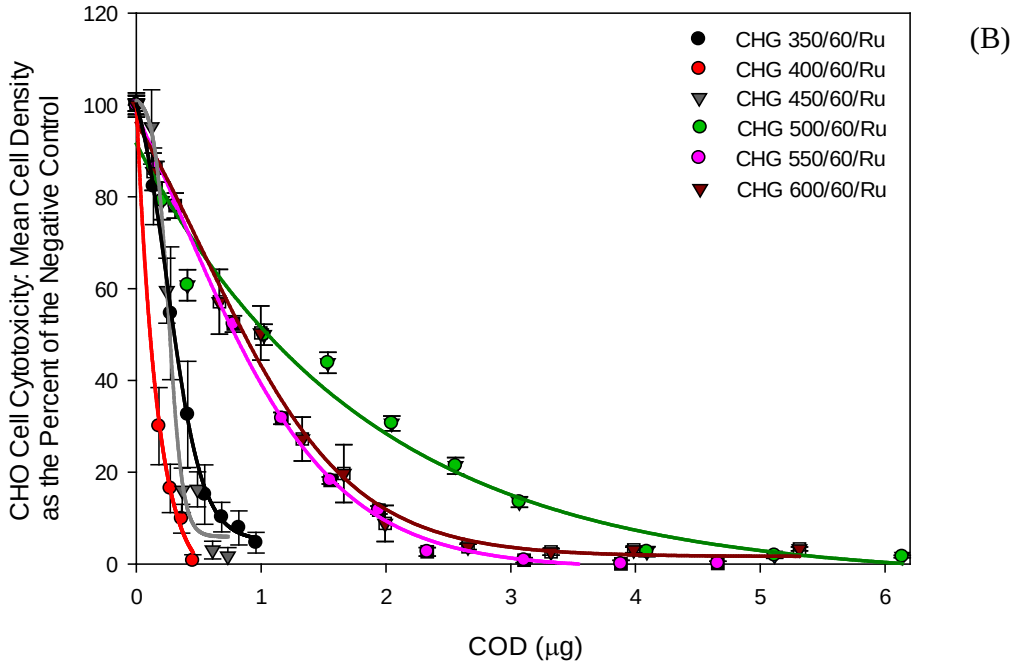


Figure 6.8 (A) CHO cell cytotoxicity index values for each CHG-WW sample (n ⩾ 3) (B) A comparison of cytotoxicity concentration response curves from CHG-WW under different temperatures (n ⩾ 8). The error bars indicate the standard error of the mean

6.3.2.4 ACUTE TOXICITY IN CHG-WW

(A) EFFECTS OF REACTION TEMPERATURE AND TIME ON ACUTE TOXICITY

The acute toxicity of CHG-WW was analyzed with the Microtox® assay, at different temperatures from 350˚C to 600˚C under 60 minutes and Ru catalyst. As shown in Figures 6.9 (A), when the temperature was increased from 350˚C to 400˚C in biomass CHG, the normalized acute toxicity index values of CHG-WW was increased from 1,228 to 4,478, which was the highest MTI value observed from biomass CHG. But, when the reaction temperature was increased from 400°C to 600ºC, MTI values were reduced which demonstrated that the removal of microtoxic compounds were proportional to the increasing reaction temperatures (r=-0.92, P=0.01). Even though, the lowest MTI value in CHG-WW was detected at the highest temperature (600°C/60minutes/Ru), increasing reaction temperature in biomass CHG showed similar performance for the CHO cell cytotoxicity (Figure 6.8 B) and microtox acute toxicity in CHG-WW, which subsequently resulted in the higher energy recovery. These results support that the control of reaction temperature in the biomass CHG can be used to mitigate the toxicity of CHG-WW and enhance the bioenergy production.

The effects of increasing reaction time in biomass CHG was explored since the reaction time is another critical parameter for hydrothermal processes (Lu et al., 2012; Susanti et al., 2011). When the reaction time was increased from 15 to 90 minutes with a fixed parameter of 400˚C/Ru, the mean MTI values ranged from 526 to 2,148 which was the highest MTI value at extended reaction time. As shown in Figure 6.9 (B), the acute toxicity potency decreased with increasing reaction times from 30 to 90 minutes because more thermal energy was available to remove toxic compounds with increased reaction times. Thus, when the reaction time was increased in HTL and

CHG of biomass, acute toxicity of aqueous products from both tests were descended and had the positive correlation each other (r=0.9, P=0.2). These results critically supported the following observations. First, the increase in reaction time and temperature was correlated to the decrease of cytotoxicity potency, which can subsequently result in the higher energy recovery. Second, reducing acute toxicity of the CHG-WW could be achieved by control of CHG operating conditions.

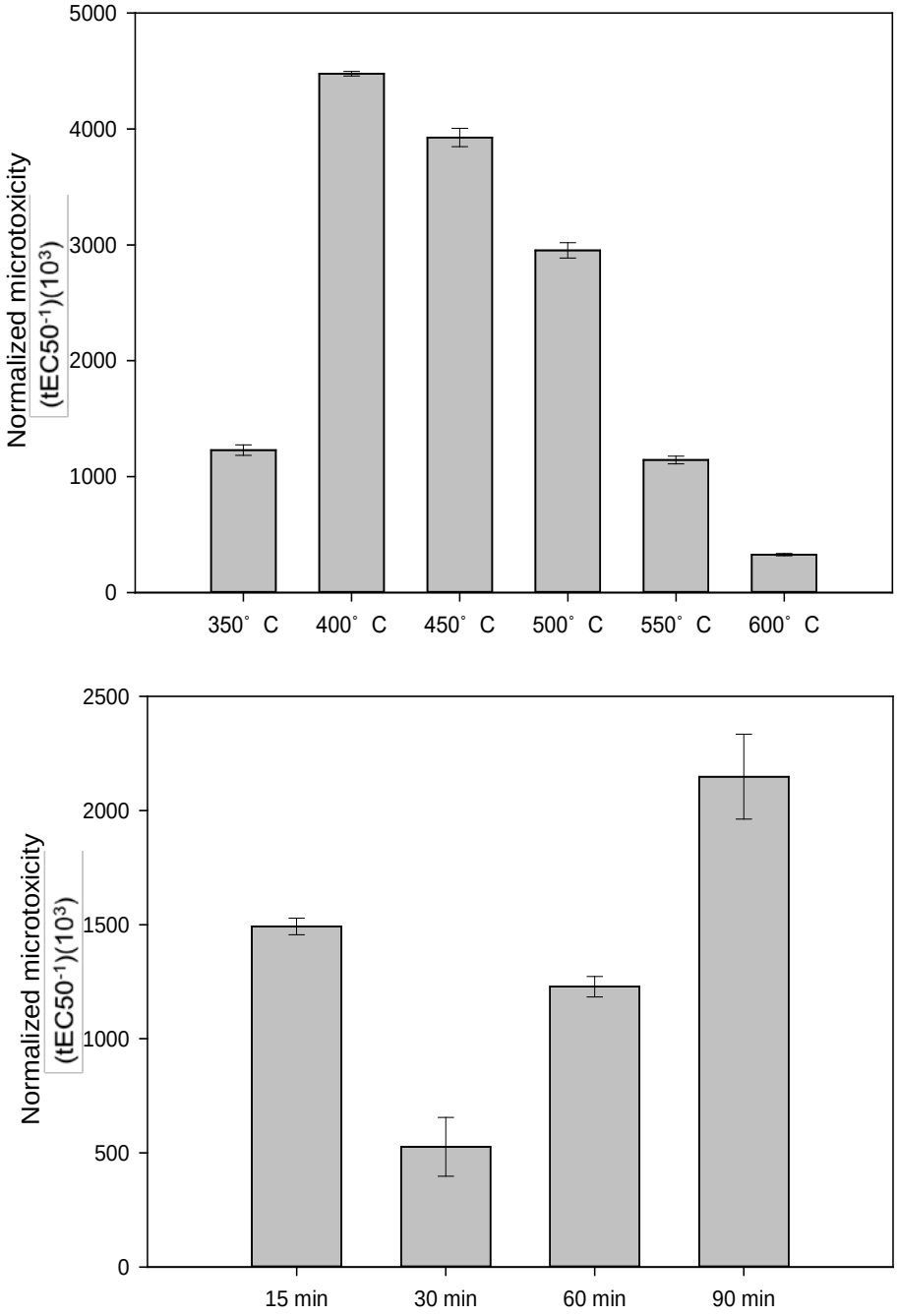


Figure 6.9 (A) Normalized acute toxicity index values for CHG-WW samples with different temperature (n ⩾ 3) (B) with different reaction time (n ⩾ 3). The error bars indicate the standard error of the mean

(B) EFFECTS OF REACTION CATALYST TYPE AND AMOUNT ON ACUTE TOXICITY

In biomass CHG, when 5 different combinations of metal and alkali catalysts were tested, the descended acute toxicity in CHG-WW was seen when Ru, Ru/NaOH, and NaOH catalysts were used, as shown in Figure 6.10 (B). The CHG-WW normalized acute toxicity index values ranged from 856 to 2,446, and the performances of the 5 different catalysts for acute toxicity followed this order: Ru > NaOH > Ru/NaOH > Ra-Ni/NaOH > Ra-Ni. The performances of Ru, NaOH, and Ru/NaOH as a metal, alkali, and mixed catalyst to reduce acute toxicity were promising, but the combination of NaOH and Ra-Ni could not contribute to enhance toxicity removal. Therefore, Ru demonstrated more effective removal of acute toxicity from CHG-WW than the other catalyst and showed the highest removal of dissolved organics from CHG-WW, which concurrently resulted in the higher energy recovery.

As the amount of Ru catalyst was increased from 0.1 to 4g per 10g biomass, the MTI values were decreased from 3,762 to 986. As shown in Figure 6.8 (A), MTI values in CHG-WW were reduced higher than 0.5g catalyst per 10g biomass with a significant correlation ($r=-0.95$, $P=0.01$). These results demonstrated that higher dose of catalyst in biomass CHG could decrease the acute toxicity in CHG-WW. Thus, according to the results in 6.6 (B), 2g of Ru catalyst could be an optimal dose for lower sCOD and acute toxicity in CHG-WW. However, the adequate dose of catalyst for biomass CHG could not be decided through these results dues to the high capacity of water absorbance, high cost, and no results for the energy recovery in biomass CHG with the various amount of catalyst.

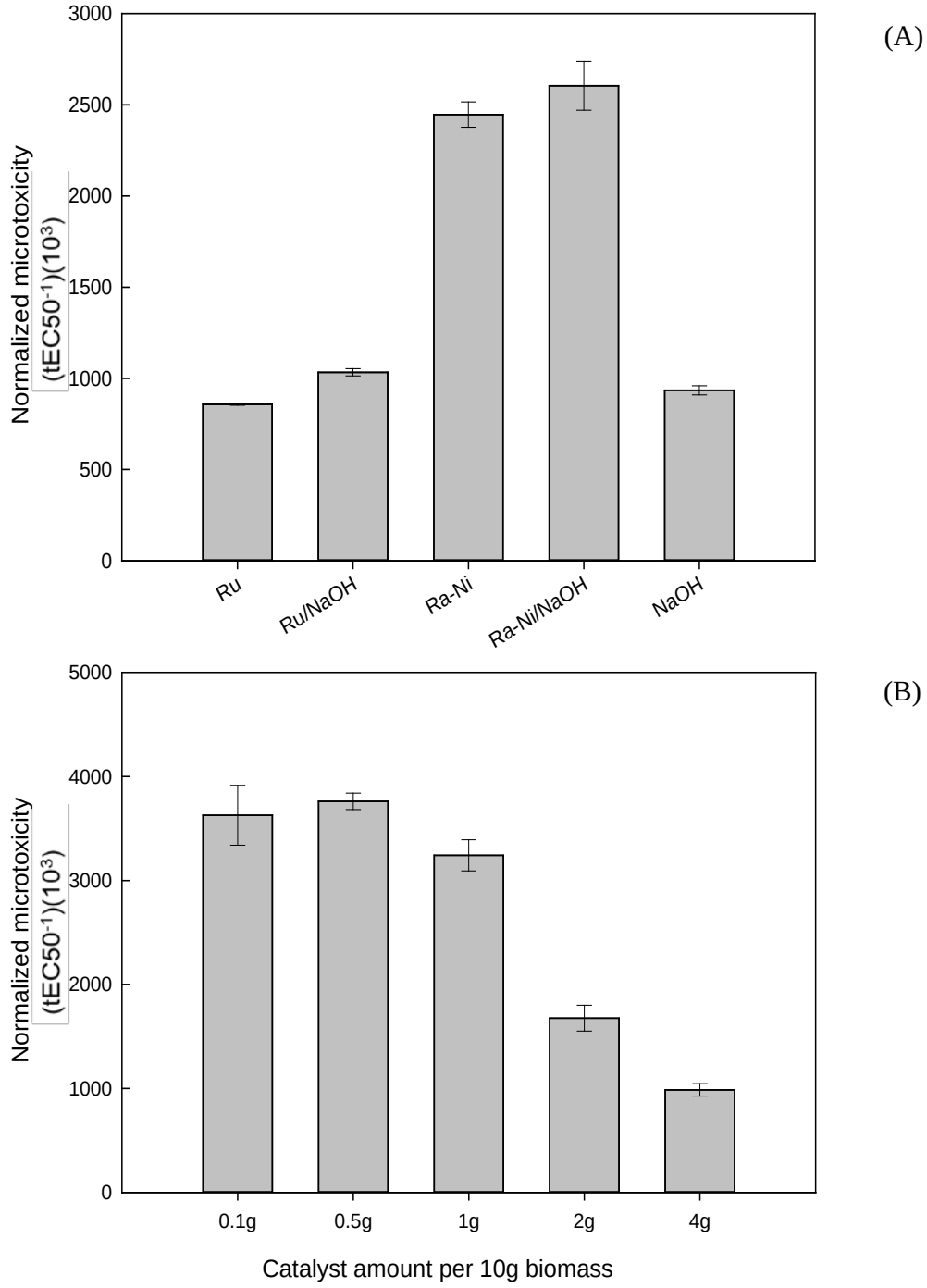


Figure 6.10 (A) Normalized acute toxicity index values for CHG-WW samples with different catalyst types (n ⩾ 3) (B) with different catalyst amount (n ⩾ 3). The error bars indicate the standard error of the mean

6.3.3 CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF HTL/CHG-WW

We evaluated the use CHG (HTL/CHG-WW) to remove residual CECs, organics, and toxic compounds in the HTL-WW that meet our target concentration for CECs (<10 ng/L) and water quality parameters related to heavy metals and toxicity. In this study, the validity of HTL-WW as a feedstock for MABB for water reuse and nutrient recycle will be evaluated based on the preliminary tests under increasing reaction temperature in CHG of HTL-WW.

6.3.3.1 WATER QUALITY ANALYSIS OF HTL/CHG-WW

The initial concentration of the feedstock has the most dominant effect on water quality parameters in the HTL-WW. During the CHG of HTL-WW, the operating parameters that can affect the water quality of HTL/CHG-WW could be different compared to biomass CHG. As shown in Figure 6.11, when the reaction temperature was increased from 300°C to 600°C, the sCOD was decreased from 35,000 to 4,400 mg/L which was the highest percent removal of dissolved organics, 87.4%. The higher reaction temperature had the negative and significant relationship with sCOD in HTL/CHG-WW ($r=-0.96$, $P=0.001$). Therefore, the sCOD removal was proportional to the reaction temperatures during the sequential HTL/CHG process.

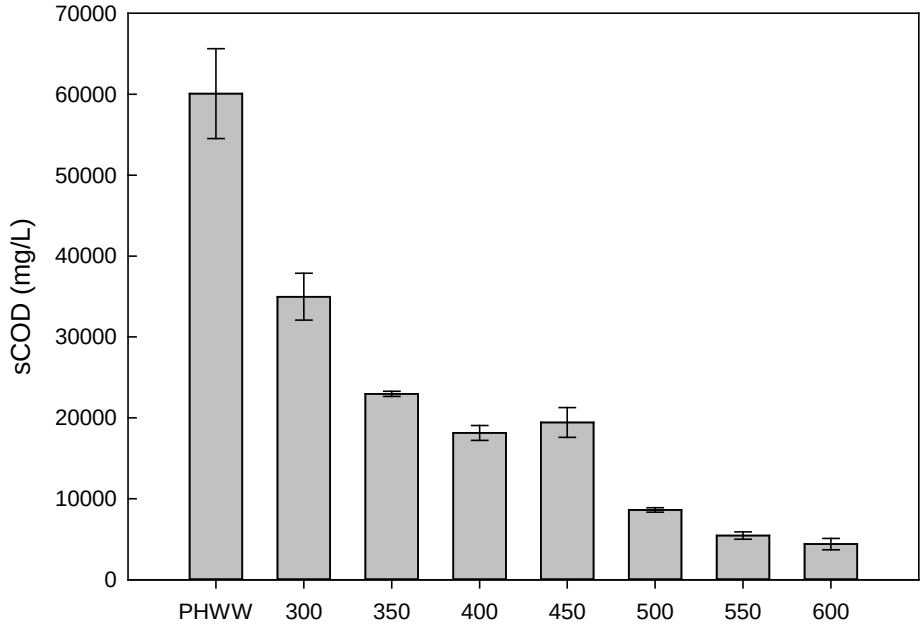


Figure 6.11 Effects of reaction temperature on sCOD in HTL/CHG-WW (n ⩾ 3). The error bars indicate the standard error of the mean

6.3.3.2 HEAVY METALS OF HTL/CHG-WW

In HTL/CHG tests, Figure 6.12 described the concentration of heavy metals in the HTL/CHG-WW and oil after the CHG of HTL-WW. The average percent distributions of metals from HTL-WW to oil and CHG-WW were 84% and 16%, respectively, and these results showed that a substantial amount of metals such as Mg, Cu, K, and Fe left to HTL/CHG-WW. Especially, the concentrations of toxic heavy metal, Cu, in the aqueous phase were 0.97 mg/L, which were higher than the suggested maximum concentrations for irrigation water (Cu: 0.2 mg/L) and livestock drinking water (Cu: 0.2 mg/L). Cu is toxic heavy metal, which could induce adverse and toxicological effects, and it is required to be monitored and removed using additional treatments (Ayers et al., 1985; McBride. 1995).

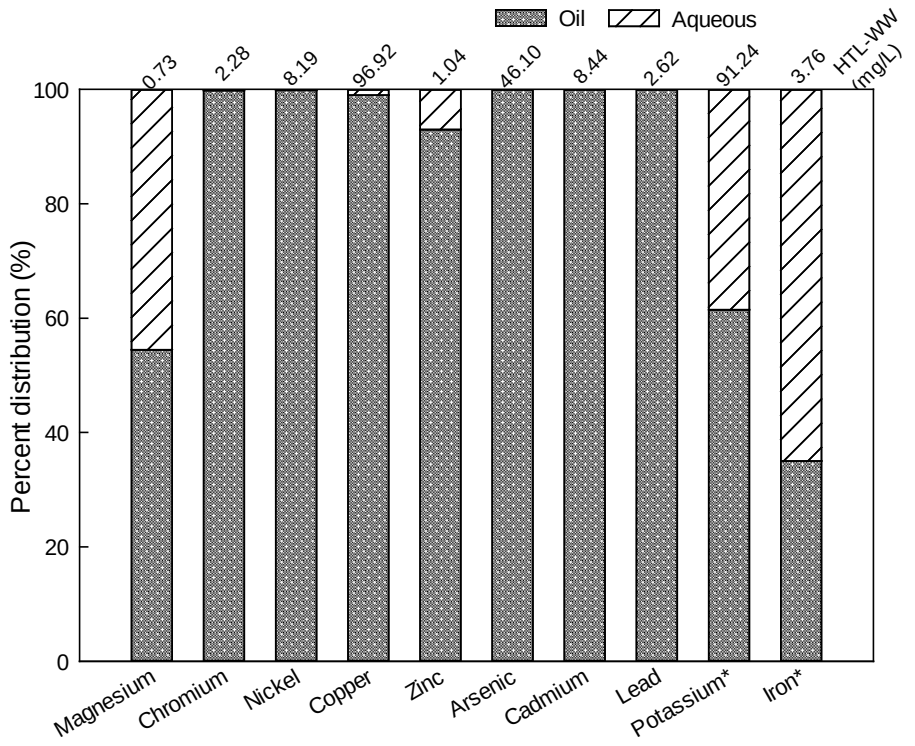


Figure 6.12 Distribution of heavy metals to the aqueous products and biocrude oil after CHG of HTL-WW

6.3.3.3 CHO CELL CYTOTOXICITY OF HTL/CHG-WW

Increasing the reaction temperature, suggested by experimental data, can potentially reduce the cytotoxic nature of HTL-WW in Figure 6.13 (A). For example, when the reaction temperature was increased from 450°C to 600°C in the CHG of HTL-WW, the original cytotoxicity index value of HTL-WW generated by the liquefaction at 300°C of a 20% solids content feedstock was 2,380, which decreased significantly from 1,367 to 349 after CHG runs (Figure 6.13 B). Because higher reaction temperatures can induce secondary and tertiary reactions in the hydrothermal medium with higher energy transfers and reaction rates, increased reaction temperatures in the CHG of HTL-WW has a negatively related to HTL-WW cytotoxicity ($r=-0.95$, $P=0.004$). Thus, increasing reaction temperature in CHG could potentially lessen the cytotoxic index of HTL-WW, showing

the highest energy recovery, 89%. Therefore, increasing reaction temperature in HTL/CHG is a favorable option due to simultaneous cytotoxicity reduction and the more energy recovery.

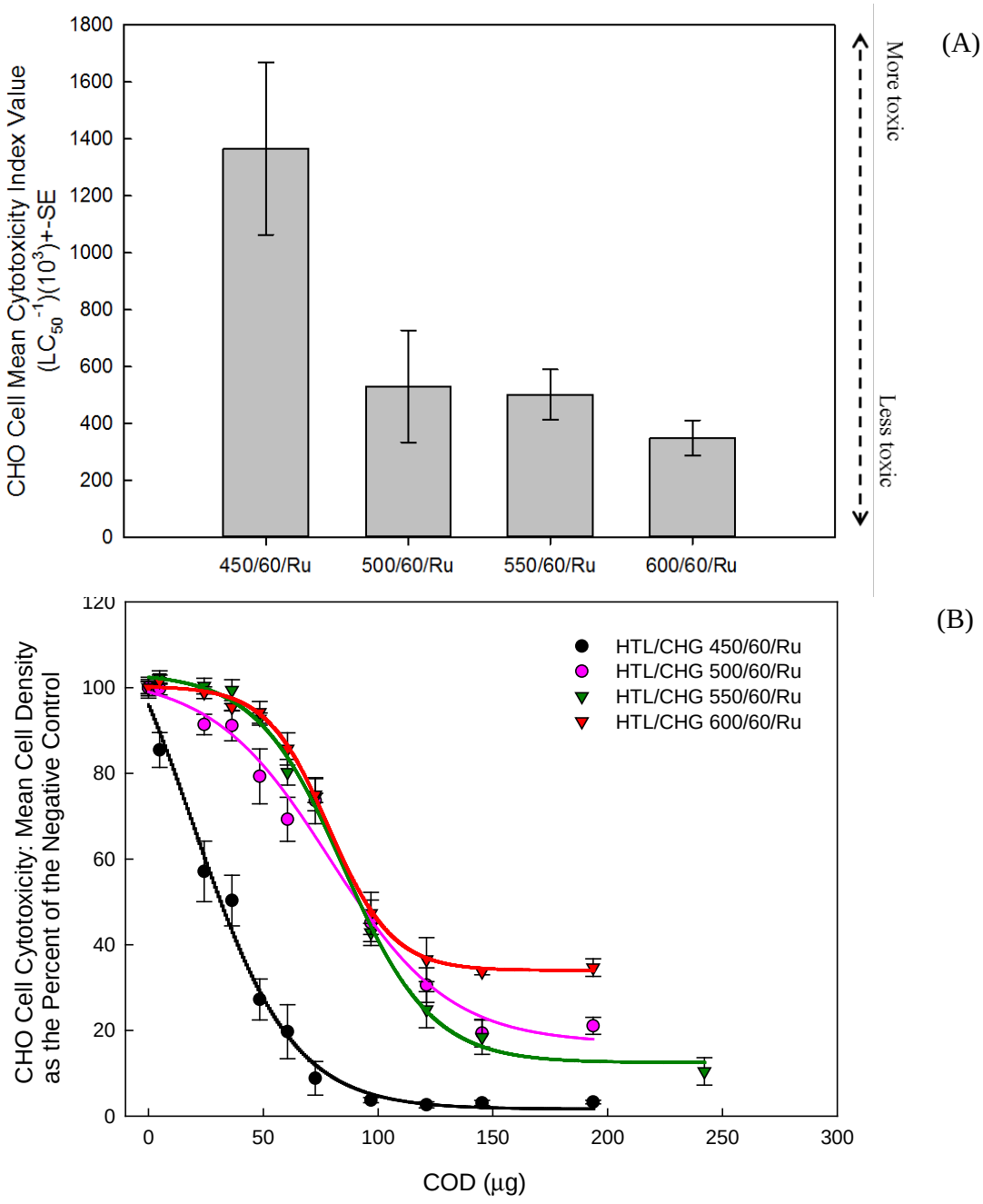


Figure 6.13 (A) CHO cell cytotoxicity index values for each CHG-WW sample (n ⩾ 8) (B) A comparison of cytotoxicity concentration response curves from CHG-WW under different temperature (n ⩾ 8). The error bars indicate the standard error of the mean

6.3.3.4 ACUTE TOXICITY OF HTL/CHG-WW

As the reaction temperature increased from 350°C to 600°C in Figure 6.14, the normalized acute toxicity index values were decreased from 2,869 to 40, which concurrently the dissolved organics (sCOD) and cytotoxicity in HTL/CHG-WW were also decreased as shown in Figure 6.11 and Figure 6.13 (B). This supports the notion that acute toxicity in HTL/CHG-WW was reduced as reaction temperatures were increased because dissolved organics including toxic compounds in HTL-WW could be generally removed by more effective conversion of organic compounds to syngas under higher reaction temperatures in CHG, which subsequently resulted in the higher energy recovery. Thus, there was a significant relationship between sCOD and acute toxicity ($r=0.92$, $P=0.01$). Therefore, lower levels of acute toxicity were detected in HTL/CHG-WW when higher reaction temperatures were used for the CHG of HTL-WW, which demonstrated the similar pattern as CHO cell cytotoxicity in Figure 6.13 (B).

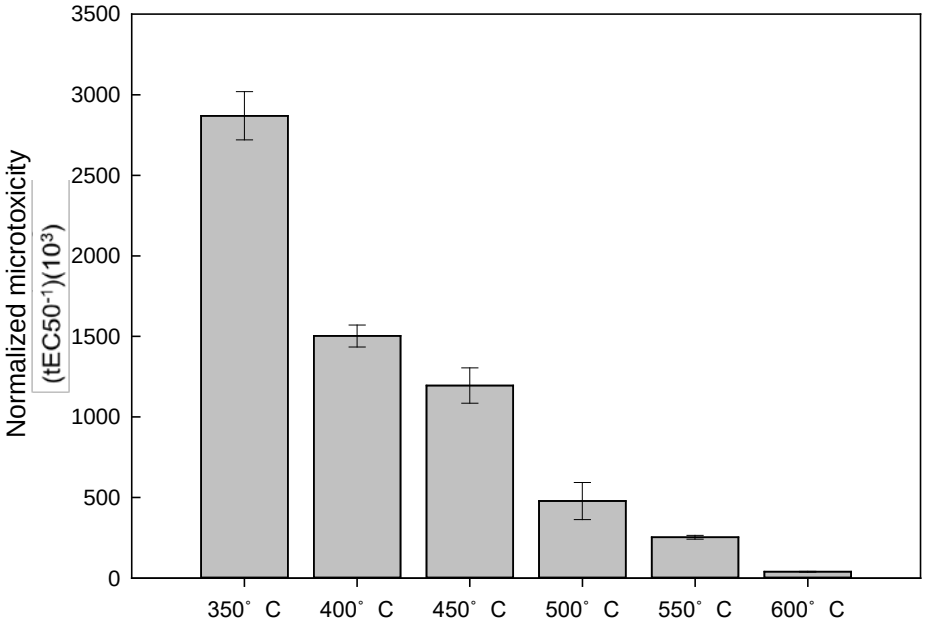


Figure 6.14 Normalized acute toxicity index values for CHG-WW samples after sequential HTL/CHG with different reaction temperatures (n ⩾ 3). The error bars indicate the standard error of the mean

6.4 CONCLUSIONS

This study investigated the effects of HTL, CHG, and HTL/CHG processes on the chemical and biological characteristics of aqueous products, where the operating conditions of each process could influence the dissolved organics, acute toxicity and/or CHO cell cytotoxicity, and heavy metals of aqueous phase.

When the reaction temperatures and times were increased during biomass HTL, the sCOD in HTL-WW were decreased, which showed that the removal of sCOD had a statistically significant correlation with HTL reaction temperatures ($r=-0.97$, $P=0.03$), but not statistically significant with reaction time ($r=-0.95$, $P=0.19$) because higher temperature in HTL could prevent the HTL-WW from increasing sCOD by inhibiting biomass liquefaction. In addition, increasing the reaction time in HTL generated higher biocrude oil yields and lower sCOD in the HTL-WW. In CHG tests, increasing reaction temperatures from 350°C to 600°C resulted in decreasing sCOD concentrations, which were shown to gradually decrease corresponding to a statistically convincing relationship with increasing temperatures ($r=-0.98$, $P=0.001$). In the sequential HTL/CHG, the sCOD removal from the aqueous phase was proportional to the reaction temperatures during HTL/CHG tests ($r=-0.96$, $P=0.001$).

To evaluate the environmental impacts of the reuse of the wastewaters from the hydrothermal waste to energy processes, the CHO cell cytotoxicity and acute toxicity were analyzed. The descending cytotoxicity potency for the increasing reaction temperature in biomass HTL was 400˚C > 350˚C > 250˚C > 300˚C ($r=0.80$, $P=0.19$). For CHG tests, the cytotoxicity index value decreased as the temperature increased from 400°C to 600°C, which toxic compounds were removed with higher temperatures under negative correlation ($r=-0.68$, $P=0.14$). Thus, the lowest CTI value in CHG-WW was observed at 500°C/60minutes/Ru, which showed the highest energy

recovery (77%, dry basis). In the CHG of HTL-WW, increased reaction temperatures had a significant and moderate relationship to cytotoxicity ($r=-0.95$, $P=0.004$) because higher reaction temperatures can induce secondary and tertiary reactions in the hydrothermal medium with higher energy transfers and reaction rates.

The microtox acute toxicity was increased with higher reaction temperature in HTL, which demonstrated a similar trend with cytotoxicity in HTL under the same conditions. As stated about cytotoxicity, the increased reaction temperature could produce more toxic wastewater by contacting oil under higher temperature (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999; Johnsen et al., 1994; Neff et al., 2006). The longer reaction times raised the subsequent acute toxicity of both HTL-WW and CHG-WW ($r=0.95$, $P=0.2$). These results support that the acute toxicity and cytotoxicity had a similar pattern for the increasing reaction temperature and time in the aqueous phase of HTL and CHG tests. When different types and amount of catalyst were applied to CHG tests, Ru as a metal catalyst was promising to reduce acute toxicity and increased amount of catalyst decreased acute toxicity in CHG-WW ($r=-0.95$, $P=0.01$). These results critically support that larger catalyst quantities and Ru catalyst effectively reduced acute toxicity and the dissolved organics in the CHG-WW. Therefore, reducing acute toxicity of the CHG-WW can be achieved by control of CHG process conditions.

The percent distribution of heavy metals to residue, aqueous, and biocrude oil during each hydrothermal process were calculated, and the metal distribution followed this order after HTL and CHG of biomass: residue (70 – 79%) > aqueous phase (18 – 29%) > biocrude oil (0.6 – 2%). These results suggest that a considerable portion of heavy metals was still present in the HTL-WW. In addition, the concentrations of toxic heavy metals in HTL-WW (As and Cu) and CHG-WW (Zn, Cd, and Pb) were higher than the recommended maximum concentrations for irrigation

and livestock drinking water (Ayers et al., 1985; EPA. 1974). This issue could potentially represent a bottleneck in the argument for the reuse of HTL-WW and CHG-WW as a potential water source, and the fate and transport of toxic heavy metals in the integrated manure management system has to be characterized as toxicological effects can be produced by these elements, negatively affecting human health and aquatic organisms (Brathwaite & Rabone. 1985; Zhou et al., 2015).

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